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TWELFTH EDITION

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12th Edition

Diseases of Poultry

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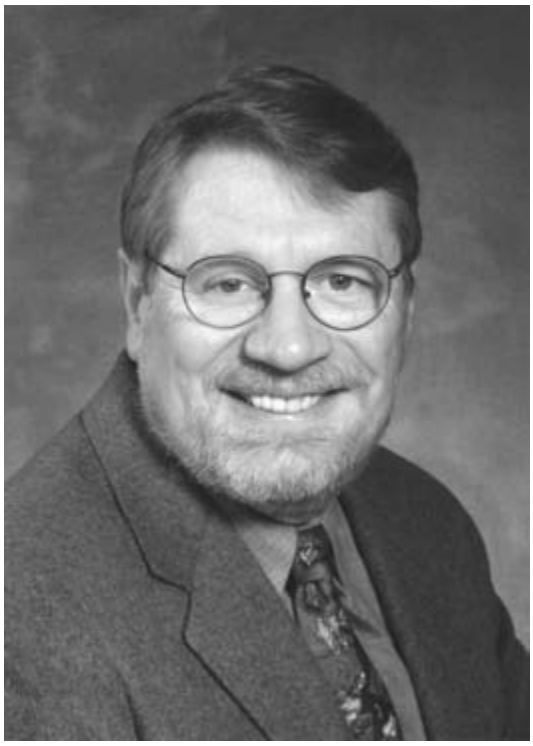
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Dr. H. John Barnes, Associate Editor,
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Introduction

The editorial board asked a distinguished colleague and former editor-in-chief of this book, Dr. Bruce Calnek, to write the Foreword to this edition containing a historical account of *Diseases of Poultry*. We are indebted to Dr. Calnek for providing a comprehensive account of the history of this book, which indeed should be preserved. Thank you, Bruce.

This edition is fittingly dedicated to Dr. John Barnes, who served on the editorial boards of the eighth, ninth, tenth, and eleventh editions of *Diseases of Poultry*. Dr. Barnes' contributions have been instrumental in maintaining the high quality of this book, and, indeed, we are highly grateful for his efforts. Dr. Lisa Nolan joined the editorial board, and we are very appreciative of her services.

This edition represents a continuation of the tradition established earlier of providing the latest information on poultry diseases. Earlier trends of expansion of authorship to include authors from around the globe continued in this edition. Efforts continued to standardize the format of the chapters to enhance the search for specific items in a given chapter.

All the book chapters were updated. There is one less chapter in this edition; some chapters were combined; and a new chapter was created. These changes were dictated by the increasing or decreasing significance of some diseases or the increasing knowledge on a given disease. Some subchapters have been moved to different chapters because of recent findings indicating that they fit within different areas.

The last chapter, "Emerging Diseases and Diseases of Complex or Unknown Etiology," has always been in a state of flux because of the nature of the topics included. Two subchapters of this chapter in the eleventh edition, labeled "Big Liver and Spleen Diseases" and "Hepatitis Splenomegaly," were recently associated with avian hepatitis E virus infection and moved to Chapter 14. In addition, the subchapter on viral proventriculitis was moved to this chapter because of the uncertainties of the etiology of the disease. The subchapter, "Poult Enteritis and Mortality Syndrome," was combined with the subchapter, "Multicausal Enteric Diseases," in recognition of the fact that it is a condition resulting from multiple infections.

Chapter 1 was split in two, and a new chapter, "Host Factors for Disease Resistance," was created. This was done in recognition of the importance of the subject matter for a book on diseases and because of the increasing knowledge on the subject. The chapter on avian encephalomyelitis was combined with Chapter 14 in which coverage of similar infections is placed. The chapter on bordetellosis was combined with the chapter on pasteurellosis and other respiratory bacterial infections.

A collective thank you goes to the authors who contributed to earlier editions of the book and those that contributed to the current edition. It has been a delightful experience working with all of you.

The personnel at Wiley-Blackwell that worked on this edition have been most helpful and accommodating, and we sincerely appreciate their support.

This is the second time that I served as editor-in-chief of *Diseases of Poultry,* and I (YMS) am indebted to my colleagues, the associate editors, for their tireless efforts in the review process and their support and advice.

Finally, I would like to acknowledge a special person, my associate Ms. Hannah Gehman. Her organizational skills, patience, speed, attention to details, and pleasant demeanor have been most helpful and appreciated.

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L.R. McDougald
L.K. Nolan
D.E. Swayne

Foreword

Previous forewords for *Diseases of Poultry*, beginning with the first one written by John R. Mohler in 1943, have briefly described the nature and contents of the edition, along with substantive reasons for its publication and distribution to potential users. He pointed out that for a profitable poultry industry ". . . knowledge of the characteristics of each disease is necessary . . . as the first step in building up an effective barrier against it." He further noted that ". . . this unusually comprehensive book is intended for students, veterinarians, pathologists, and workers in specialized fields." These words are as applicable today as they were 65 years ago.

For the sixth edition in 1972, Dr. P. P. Levine offered an accounting of some of the changes in the poultry industry that moved it from a small-scale farm activity to ". . . a highly sophisticated industry marketing products worth over $6 billion per year in the United States alone." He correctly attributed some of the many advances in disease control through eradication, genetic selection, immunization practices, management improvements, and so on to major advances founded in research. Such new knowledge strongly dictates a need for revised texts. Levine further predicted that ". . . infectious diseases will decline in importance; toxicologic, nutritional, genetic, and husbandry problems will demand increasing attention. Change is the order of life, and avian diseases are no exception." In the seventh edition (1978), he pointed out many of the important new advances in identifying the etiology of several conditions, and the need for *Diseases of Poultry* to ". . . keep up with the rapid developments in avian diseases."

Ben Pomeroy, in the eighth (1984) and ninth (1991) editions, reiterated the need for new editions to keep up with the ". . . explosion of knowledge on the prevention and control of avian diseases." The inclusion of contributions from experts from many countries of the world and the importance of such in the face of global issues of disease control were emphasized by Charles Beard in the tenth (1997) edition. He pointed out that understanding the molecular genetics of causative agents is also important and that the use of molecular methods is necessary for poultry disease researchers to understand and control infectious diseases; yet another reason for timely updates.

The message is clear: a changing and global poultry industry and its many allied industries need the most recent information available to keep pace with the challenges of providing adequate health care and disease prevention. It is important not only to poultry flocks, but also to the consumers who expect safe, as well as nutritious, poultry products. This, the twelfth edition, upholds the long-standing reputation of this book for keeping scientists, breeders, poultry producers, and poultry health professionals supplied with the latest and most comprehensive information available.

Sixty-five years have passed since the first edition was printed. Before all details are lost forever, it is fitting to look back at how this "bible" in the field of poultry diseases came to be and how it has evolved into what it is today. It all began in the 1930s. In a memorandum addressed to the American Association of Avian Pathologists (AAAP), dated December 22, 1965, H. E. Biester related the events that preceded the decision by the Iowa State College (now University) Press (ISU Press) to undertake the publication of *Diseases of Poultry*. During the 1930s, Louis DeVries, a member of the Department of Modern Languages at the college, translated a 1929 German book entitled *Handbuch der Geflügelkrankheiten und der Geflügelzucht*, published by Ferdinand Enke, Stuttgart. The translation lay dormant for several years until Dr. Campbell, the Chicago publisher of *Veterinary Medicine*, saw the translation and expressed some interest in it. Dr. Biester, who described himself as an "innocent bystander, having no special interest in the project" told Dr. Campbell that the manuscript was unacceptable for a variety of reasons, and he suggested that if he were serious about publication, then selected specialists should edit or rewrite the material. Dr. Biester later was pulled into the project, and he ultimately concluded that the German book was obsolete. Apparently, a number of contributors had accepted invitations to cooperate in developing an American book, and, according to Biester, they agreed that ". . . it would be better to prepare a totally new book based on American conditions."

Thus, the die was cast. Dr. Campbell gave up his plans, and ISU Press decided to publish an original text. Drs. Biester and DeVries served as editors, and 34 American investigators were engaged in the project. There were chapters on general subjects such as anatomy, digestion, genetics, hematology, hygiene and sanitation, nutrition, and surgery as well as those dealing with specific infectious and noninfectious diseases and conditions. A separate chapter dealt with diseases of turkeys. In 1943 the first edition was ready. The publication costs were considerable for a book that was thought to have limited distribution, so it was decided to omit royalties and accept a subsidy for illustrations from the dean of the college. Fifteen hundred copies were printed and placed on sale for $7.50. To everyone's surprise, a second printing of 2,500 copies was needed after less than nine months, and there was yet another printing of 2,500 copies two years later.

Royalties were then instituted! ISU Press was concerned that without some remuneration, the authors might be reluctant to remain "dedicated."

The inclusion of Dr. DeVries as an editor is a bit puzzling since he had no medical background. Perhaps it was in recognition of his effort with the translation of the German text. In any case, he was replaced in subsequent editions by Dr. L. H. Schwarte, a member of the Veterinary Research Institute in Ames who had written four chapters in the first edition. The book was thereafter referred to by many as "Biester and Schwarte" even for a period after they were no longer associated with it. They continued at the helm through the fifth edition published in 1965. Although Dr. Schwarte contributed several chapters in each of the first five editions, Dr. Biester apparently confined his efforts to editorial tasks. Their memo to the AAAP stated that they both were responsible for making the index, and they personally checked practically all of the references because they felt that they "owed to the reader accuracy." A total of 61 people served as authors under their editorial supervision; 12 of them contributed to all five editions.

Ultimately, the passage of time dictated that Drs. Biester and Schwarte should relinquish their roles as editors, and they decided that the fifth edition (1965) would be their last. As noted in the preface to the sixth edition, it was their wish ". . . that future editions of the book become the responsibility of the AAAP. . . .", which had become a strong and representative organization to which many of the users of *Diseases of Poultry* belonged. Also the AAAP was already in the business of publishing the journal *Avian Diseases*, so it was considered a logical move. The AAAP appointed a committee, chaired by Dr. M. S. Hofstad who had been one of the book's authors and who was on the faculty in Ames. Drs. Biester, J. E. Williams, B. S. Pomeroy, and C. F. Helmboldt filled out the committee, and, in June 1966, they recommended that the AAAP sponsor future editions of *Diseases of Poultry,* which would continue to be published by ISU Press in Ames. They asked the board of directors to appoint an editorial committee consisting of an editorial chairman and four associate editors by January 1, 1967. A letter from Dr. G. H. Snoeyenbos (AAAP secretary-treasurer) to Dr. C. A. Bottorff (AAAP president) dated November 23, 1966, suggested that Dr. P. P. Levine had declined a proposal that he assume the editorship of the book. Dr. Hofstad was subsequently named editor, and he personally requested that Drs. Helmboldt, B. W. Calnek, W. M. Reid, and H. W. Yoder, Jr., be invited to be the associate editors. Each was given responsibility for a group of chapters that largely represented their individual interests and strengths. An agreement between the AAAP and the ISU Press was executed on May 8, 1967, and it was agreed that manuscripts would be delivered to the publisher by September 1, 1969. So the transfer was complete and official.

The sixth edition, under totally new editorial support, underwent some significant changes. The length of the book was beginning to be of concern and there was some discussion about perhaps needing to split it into two volumes. To avoid this, several chapters (anatomy, nutrition, genetics, and hematology) were eliminated based on good coverage in other publications. Also, there was a consolidation of other material; for instance all neoplastic diseases were placed in a single chapter, and turkey diseases were incorporated in other chapters based on etiology. There were sweeping changes in authorship. Only 14 of the 40 contributors to the sixth edition had participated in the fifth edition. Clearly, a new era had arrived!

Also, following concern for the book's length, Dr. Hofstad asked that the number of listed references be reduced by *selective citation.* He agreed that the reader should *find*, or *be directed to*, all pertinent literature on each of the covered topics, the latter through citation of review papers, etc. Space allocated to references became an issue in subsequent editions as well. In the seventh and eighth editions, titles of all references were removed. This was controversial and not all editors agreed—including the author of this review—and reference titles appeared again beginning with his tenure as editor of the ninth edition. Interestingly, based on the number of pages, the third edition (1,245 pages) was actually longer than the eleventh (1,231 pages). But by increasing page size, decreasing type size, and splitting into two columns per page, it was possible to include more than twice the amount of written material in the latter.

Unlike the situation with Drs. Biester and Schwarte, citations and their accuracy became the responsibility of the individual authors. When it was observed that many errors existed, authors of the ninth edition were asked by Dr. Calnek to check every single reference against the original work so as to assure accuracy. This met with an enormous number of groans and considerable resistance until each author (with perhaps an exception or two) followed this strict instruction. The subsequent turn-around in their attitude was truly amazing when nearly all of them found errors, including the citation of references that did not even exist. It was not uncommon to detect mistakes in as many as 10 percent of citations in some chapters, probably due in large part to a common practice of copying reference citations from other lists.

Beginning with the ninth edition, the book entered the electronic age. All material was submitted or copied into a word processing program that allowed spell-checking and reformatting. Initially, it was a tedious job, particularly because personal computers at that time were slow, and the skill of the individual authors in mastering a new approach varied considerably. However, improvements in software and computers and the possibility of rapid transfer of texts between authors, editors, and the publisher made the preparation of a new edition pleasurable compared to the old "hard copy" approach.

There has been a continuum of changes that have improved *Diseases of Poultry* and kept it relevant over the years. For the tenth edition, the editors carefully reviewed and upgraded illustrations, and, for the first time, included a number of color plates. Another major improvement that was gradually incorporated was the inclusion of molecular biology in many of the chapters. This was particularly important with regard to new applications of molecular techniques in diagnostic procedures, descriptions of etiological agents and significant elements of their molecular makeup, understanding the significance of selected genes in the pathogenesis of the diseases, and the development of genetically engineered vaccines. Our understanding of the fundamental na-

ture of many diseases is now founded on the use of molecular approaches in the research laboratory.

Another of the more significant evolutionary changes was the addition of foreign authors to make the book truly international in flavor. One of the original AAAP-appointed editors argued strongly that *Diseases of Poultry* should be an "American" book, and the authorship was so aligned. The sixth edition had the first "foreign" contributor, although he (Bela Tumova, from Prague, Czechoslovakia) was actually a visiting professor at the University of Wisconsin working with B. C. Easterday on avian influenza at the time. It wasn't until the eighth edition that invitations to contribute to the book were extended to workers outside of the United States. Drs. P. M. Biggs and L. N. Payne from England and Drs. J. B. McFerran and M. S. McNulty from Northern Ireland thus paved the way by providing parts of the chapters on neoplastic diseases, adenoviruses, and miscellaneous viral infections. The next edition (ninth) was truly international with 17 contributors from 9 countries outside of the U.S., and by the eleventh edition, there was a total of 34 different contributors from 13 countries. The world-wide reputation of the book was certainly enhanced by the selection of authors based on their knowledge and contributions to our understanding of individual diseases and conditions without regard to their geographic location.

The importance of *Diseases of Poultry* as a text for the world also is reflected in its translation into foreign languages or publication in a "copied" form in other countries. There have been several authorized translations into Spanish, Chinese, and Russian, and an agreement between the publisher and India has allowed what is essentially a photocopied version of the original to be made.

An ongoing review of the relative importance of individual diseases or conditions has led to a good deal of reshuffling over the years. Chapters have been added, combined, split, or eliminated to meet the changing picture of what is important to the field of avian diseases and disorders. Periodically, and especially with a change in authorship, major rewriting of some sections takes place. New chapters such as one dealing with new and emerging diseases appear when needed.

Changes in editors occurred over the years so that by the eleventh edition, none of the 1968 group appointed by the AAAP remained. After riding herd on three editions (sixth–eighth), Dr. Hofstad retired and was replaced by Dr. Calnek (ninth and tenth), and he, in turn, passed the baton to Dr. Y. M. Saif beginning with the eleventh edition. Likewise, associate editors who have replaced or been added to the original group appointed by the AAAP in 1968 include Drs. H. J. Barnes (eighth–eleventh), C. W. Beard (ninth and tenth), L. R. McDougald (tenth), Y. M. Saif (tenth), J. R. Glisson (eleventh), A. M. Fadly (eleventh), D. E. Swayne (eleventh), and Lisa K. Nolan (twelfth).

In summary, it is obvious that the "bible" in the field of avian diseases is an evolving, vibrant, and ever-current source of information relevant to all practitioners in the field of poultry medicine. It continues to be a reference source of significance to a vast number of people with many different relationships to the poultry industry.

Bruce W. Calnek

12th Edition
Diseases of Poultry

12th Edition

Diseases of Poultry

Chapter 1

Principles of Disease Prevention: Diagnosis and Control

Introduction

Alex J. Bermudez

This chapter acquaints the reader with the general concepts of poultry disease prevention, diagnosis, and treatment. It specifically introduces the reader to management practices used in disease prevention and poultry vaccination, principles of antimicrobial therapy, and basic necropsy procedures. This chapter also provides information on insecticides and disinfectants. For information on specific diagnostic techniques and control measures, the reader is referred to the respective chapters covering specific diseases in this text.

This chapter does not cover all the detailed disease control methods or all types of poultry but attempts only to outline and illustrate some fundamental concepts. Each poultry enterprise is different; therefore, these basic concepts must be applied according to conditions and facilities existing in individual situations. To keep abreast of the flow of research and information, a constant review of current literature and recommendations applicable to specific diseases, special enterprises, and various geographic areas is necessary. Excellent journal sources of current information are *Avian Diseases*, *Avian Pathology*, *Poultry Science*, *Journal of Applied Poultry Research*, and *World's Poultry Science Journal*, and many publication and trade journals provide special emphasis on particular segments of poultry husbandry (e.g., *Poultry International*, *International Hatchery Practice*, *Industria Avicola*, *Egg Industry*, *Watt Poultry USA*, *International Poultry Tribune*, *Poultry Times*, and others, including publications in a variety of languages). Standard textbooks on chicken and turkey production, husbandry, and nutrition are other sources of information. A good practical manual on commercial chicken production is North and Bell (2).

During the last three decades, the poultry industry has experienced many changes that have had a significant effect on the prevention and control of poultry diseases. One of the most significant changes has been the continued integration of poultry production companies and the consolidation of these companies and the allied industries that serve them. These changes have a profound effect on the poultry industry as the decision-making process becomes more centralized in poultry companies and fewer products and services become available with the mergers of biologic and pharmaceutical companies.

While the poultry industry has changed significantly as the result of internal market forces, it also experiences significant external forces that are largely beyond its control. Examples of such forces include the globalization of the world economy, food safety issues, environmental concerns, animal welfare issues, and concerns about the use of antimicrobials in food animals. These external forces have a very direct impact on the activities of poultry health professionals as they certify poultry or products for export, address consumer concerns, and work to ensure the production of safe and wholesome poultry products. Although many of these topics are beyond the direct scope of this text, they can have a significant indirect or direct impact on the disease agents that affect poultry.

Host-Parasite-Environment Relationship

Disease results when normal body functions are impaired, and the degree of impairment determines the severity of the disease. It may result from the consequences of harmful actions of infectious and parasitic agents, or it may be caused by injury or physical stress with which the bird cannot cope. Disease may also occur as the result of a deficiency of a vital nutrient or the ingestion of a toxic substance.

Diseases caused by infectious and parasitic agents are frequently complex and depend upon characteristics of the host, agent, and environmental conditions on the farm. Nutritional deficiencies may be temporary and reversible when the nutrient is supplied in adequate amounts; others are irreversible. Disease resulting from stress is related to its severity and duration. Injuries, such as extreme beak trimming, tend to persist for a long time and may be permanent.

Whether disease results from parasitism depends on the number, type, and virulence of the parasite; the route of entry to the body; and the defense status and capabilities of the host. The latter depends partly on the host's prior disease encounters (e.g., infectious bursal disease or IBD), nutritional status, and genetic ability to organize resistance mechanisms; environmental stresses; and the kind and timing of countermeasures employed (drugs or changes of environment).

Some virulent organisms, such as highly pathogenic avian influenza, rapidly overcome the resistance of even the healthiest hosts. Less virulent strains or types cause moderate to severe ill-

ness, but most birds respond and return to a state of health. Still other strains or types cause no marked reaction, and the host shows little or no obvious signs of ill health. Some infectious agents may not cause dramatic effects themselves but predispose the host to more serious infections by other agents. Some microorganisms are not considered pathogenic because they usually are found in and around individuals considered "normal;" it must be recognized, however, that so-called nonpathogenic and low pathogenic organisms can also cause serious losses when the right environmental circumstances exist. Severe physical stresses such as chilling, overheating, water deprivation, starvation, and concurrent infection by other disease agents can reduce the host's ability to resist and, thus, may precipitate a disease condition that can be detected (e.g., clinical mycoplasmosis following infectious bronchitis or clinical salmonellosis in chilled or water-deprived chicks).

Coccidiosis provides a good example of the relationship between the number of invading organisms and the severity of the resulting infection, because the morbidity and mortality of the host species are usually proportional to the number of sporulated coccidial oocysts ingested. Environmental conditions still play a significant role, however, as litter conditions will affect both the rate of oocyst sporulation and survival. A similar situation exists for many other infectious diseases. A mild roundworm infection may not be serious; whereas a severe infection can be very detrimental. A good reason for removing moribund and dead birds from a flock is to reduce the number of infectious organisms available to penmates. Thorough washing and disinfecting of a building may not render it sterile, but it can reduce the number of infectious organisms to such a low level that they cannot cause disease.

By following sound disease-preventive practices before and after the arrival of new flocks; making sure that the flock has adequate, properly placed, good quality feed and water; applying judicious and timely vaccines and medications; and providing a less stressful environment, the poultry producer can control the probability of a flock becoming infected, as well as the severity and outcome of an infection.

Influence of Modern Practices

Avian disease specialists continually must seek new knowledge about the nature and control of specific diseases. Meanwhile, persons responsible for the production of poultry meat, table- and hatching-eggs, chicks and poults, feed ingredients, and mixed feeds should practice the basic techniques and management principles that will prevent occurrence of disease. They should also provide the physical facilities and quarantine capabilities necessary for control and elimination of diseases that occasionally gain entrance so that they do not become a continuing problem. Economic losses, sometimes relatively subtle, resulting from disease can mean the difference between success and failure in the poultry business. Those who disregard the basic principles of disease prevention may succeed in times of a favorable market but do not remain competitive when the margin of profit is very small. A new modern enterprise with many good buildings and labor-saving equipment, but constructed and operated without regard to fundamental disease control and eradication principles, may function free of disease for a few years. All too frequently, a troublesome disease gains entrance and thereafter becomes a constant costly burden because of the extreme cost of depopulation required to eradicate it.

When new farms and buildings are designed and constructed and production is programmed with the objective of excluding diseases or eradicating them when they gain entry, poultry can be maintained free of most harmful diseases in a practical manner with reasonable effort. The poultry producer who uses fundamental management practices that prevent disease outbreaks has little need for detailed knowledge of the many infectious diseases affecting poultry.

Facilities need not be new to be adequate. Frequently, old farms can be enlarged and production reprogrammed to exclude or eradicate disease. Many old poultry buildings, hatcheries, and feed mills can be redesigned to favor exclusion, eradication, or control of disease. Strict application of disease-preventive management techniques has enabled producers to maintain specific-pathogen-free chickens on farms of standard design and construction (1).

The trend in all agricultural industries continues toward larger units, fewer farmers, and corporate enterprise. The chicken and turkey industries have been leaders in this trend, which has placed emphasis on efficiency and lower costs of production. Survival in the industry has depended upon continual adoption of newer and more efficient practices. It is sometimes forgotten that efficiency in disease prevention is as important as efficiency in cleaning, feeding, bird handling, and egg processing. The resulting evolution of management systems has altered the emphasis in disease-control practices and will continue to do so (e.g., the shift in placing layer flocks in multi-flock complexes rather than single-age farms has eliminated the possibility of all-in, all-out production and provides a much greater challenge to the flock manager in the control of respiratory disease).

Corporation farming accelerated the move toward integrated control and operation of two or more segments of the industry, such as feed manufacturing, breeder flock management, hatchery operation, pullet rearing, broiler and turkey grow-out phases, laying farm production, egg processing, turkey and broiler slaughter and processing, retail distribution, and further processing of poultry products. Integration of the industry has concentrated under one decision-making body the disease control practices for millions of birds, as well as several phases in the production chain of eggs and meat. Thus, sound health practices and emergency quarantine measures decided upon by one or a few individuals can be applied quickly and effectively to large numbers of birds. Through integration, it has become economically practical to employ veterinarians full time and to place responsibility for disease control directly in the hands of specialized poultry veterinarians. Disease considerations are sometimes reduced to simple cost accounting, whereby the economic loss from a disease and the costs of treating it are weighed against the costs of its eradication and of maintenance of the clean status, before determining the course of action. Poultry production managers must be careful not to make decisions that produce short-term cost savings

but potentially greater negative long-term cost effects as the result of a higher disease incidence. Examples of such potential shortsighted cost savings are the excessive dilution of Marek's disease vaccine, reusing litter in turkey brooding facilities, and excessively shortening the turn-around time between production flocks.

The poultry industry can no longer be considered to be composed of localized businesses limited to certain states or areas. It is characterized by multistate and often multinational companies that move products daily between widespread locations and markets. Because of the high cost of scientific poultry breeding, producers throughout the world depend on a few organizations for their highly efficient breeding and production stocks. In the case of turkeys, most of the world's breeding stock originates from one of three locations in North America. For such a system to function smoothly and efficiently, widespread and daily shipments of hatching eggs, poults, chicks, started pullets, and adult fowl across state and national boundaries are essential and necessitate reevaluation of old concepts of health regulations. Specialized poultry veterinarians and state and federal livestock health officials have evolved to guide the course of health control measures. Diagnostic facilities, both private and government, are available in major poultry-producing areas of the world. Except where importation and use are restricted by government regulation, high-quality vaccines and drugs are available wherever poultry is raised commercially. Good quality feed is the rule, not the exception, in the modern poultry industry.

Despite significant advances, disease still takes a heavy toll from all types of poultry enterprise. Those who exercise farm management decisions (caretaker, owner, flock supervisor, corporate manager, money lender) have the power to reduce these losses through management for disease control. They must be made aware of the responsibilities and continually encouraged to develop a philosophy of disease prevention through management and to concentrate on amortized long-term advantages and not just short-term savings.

With better control over diseases of all kinds, providing optimum bird comfort throughout the house has become a very important management factor in obtaining maximum performance. That is not achieved solely by windowless, insulated, light- and temperature-controlled houses. Such factors as overcrowding, poor beak trimming, uneven temperatures, and uncomfortable air currents on caged birds that cannot move to a more comfortable location also adversely affect performance. Proper orientation of feeders, waterers, and light promotes good performance; slight, seemingly insignificant changes from proven systems can have a pronounced adverse effect on performance of both caged and floor-housed flocks of chickens and other commercial fowl. Poor performance of adults is often traceable to detrimental events that occurred during the rearing period. An attentive and skilled farm manager is of great importance in the successful production of poultry flocks.

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Disease Prevention and Diagnosis

Alex J. Bermudez and Bruce Stewart-Brown

Breeder Flock Management

The breeder flock must be managed in such a way so as to optimize the production of clean fertile hatching eggs in an economic fashion. This management program must ensure that the chicks or poults produced will be viable from both an immunologic and nutritional perspective when they are placed in the production setting. The science of effective breeder flock management is somewhat beyond the scope of this text, and a reference on this topic has been published by Leeson and Summers (34). Disease prevention measures must also be in place to prevent diseases that will result in morbidity and mortality in the breeder flock itself. Finally, the breeder flock should be managed so that egg-borne diseases are prevented by whatever techniques are available.

Diet, Health, and Parental Immunity

A breeder ration must contain a higher level of many nutrients than does a laying ration. Laying rations sufficient to sustain egg production are not adequate to sustain good hatchability and health of young offspring. In some cases, production is satisfactory in breeder hens, but their embryos or chicks show symptoms and lesions of vitamin deficiency. The breeder ration must be adequate for development of the embryo and the chick as well as egg production performance of the breeder hen. The minimum nutritional requirements of breeder flocks are well characterized in the *Nutritional Requirements of Poultry* (44) published by the National Research Council. Poultry nutritionists responsible for the nutrition of commercial breeder flocks frequently supplement the nutrient inputs of breeder flocks beyond these minimum requirements to provide an additional margin of safety in the ration.

Meat birds are bred to grow fast and large, but the flocks kept for breeding must have feed intake restricted to prevent obesity and poor adult performance. Feed restriction must be carefully controlled to prevent aggressive birds from getting most of the available feed. Two systems are widely used: daily restriction and skip-a-day feeding. The former requires special feeding equip-

ment or procedures to ensure that feed is presented to the entire flock simultaneously so that all birds begin eating at the same time. In the skip-a-day system, feed is given in larger quantities on alternate days, permitting the recessive birds to obtain their share even if they have to wait their turn to eat. In either system, special provision must be made in formulating feeds to provide adequate coccidiostat and essential nutrients in the reduced amount consumed.

Breeder hens in poor health for any reason frequently fail to supply the embryo with some vital nutritional factor(s) or perhaps pass some toxic material to the egg; thus, the hatch is poor or chicks are of low quality and must be culled. A good example of such a problem is breeder hens infected with capillaria worms; they may produce progeny that are deficient in vitamin A. While this occasionally happens with apparently healthy birds also, a clinically healthy breeder flock is the best insurance of good quality offspring.

Young poultry are delivered into many types of environments. In some areas, husbandry methods are such that birds are exposed to disease from the first day of life. In some cases, exposure of very young poultry lacking any maternal antibodies to a disease can lead to significant mortality or economic loss (infectious bronchitis, avian encephalomyelitis, IBD, or duck virus hepatitis). Where exposure is apt to occur at a very early age, maternal antibody can be a significant aid to prevention of disease. However, a high level of maternal antibodies can interfere with early immunization. How much maternal immunity is desirable and against how many diseases are debatable subjects and will vary according to the area where poultry are raised and the type of rearing facility (cage versus floor).

Maternal immunity is dissipated gradually and usually does not last more than 2–4 weeks after hatch. In modern, well-run layer and breeder replacement-rearing facilities chicks and poults are well protected, not only against the elements, but also against introduction of disease from outside sources for several weeks or beyond the time that a high initial maternal immunity would be protective. Maternal immunity in chicks is of less concern in such cases. This is not so likely to be true of inadequately sanitized and poorly managed pullet-rearing or broiler grow-out farms, where exposure can occur as early as the first day of life to a disease agent carried over from the previous brood in reused built-up litter. In these cases, protective maternal antibodies become a very important consideration in preventing disease or reducing losses, and vaccinating breeder dams with killed vaccines to give high maternal antibody protection for the offspring has become common practice. Lesions and residues from the carrier for killed vaccines injected into the breast muscle have been cause for carcass condemnation at slaughter.

Interior Egg-Borne Diseases

Various techniques are used for preventing disease agents from being transmitted from dam to offspring via the egg. The ideal situation is to have breeders free of all pathogens. For most viral diseases, there is still no practical way of obtaining this utopian situation. For others (avian encephalomyelitis), the probability of the infection occurring during the egg-laying period, with resultant egg transmission, is too great to permit the clean but susceptible status (see Chapter 14).

Immunization

In addition to immunization of breeders against several common diseases to prevent adverse effects of inopportune infections on egg production, they are immunized against avian encephalomyelitis during the growing period to ensure that they do not become naturally infected during the period they are producing hatching eggs. Although this may not be an absolute guarantee against egg transmission of the virus, it has been a practical means of preventing its serious dissemination through infected offspring.

Testing and Removal of Carriers

Carriers of some transovarially transmitted diseases can be detected by serologic or other means, and this procedure has been used to eliminate possible egg shedders from breeding flocks. Historically, this has proved to be an important starting point in the successful eradication of pullorum disease and fowl typhoid. Similar programs currently are practiced by primary breeder companies to reduce the vertical transmission of lymphoid leucosis.

Testing and Slaughter of Infected Flocks

Where infected breeders are detected, the entire flock may be destroyed. This method is indicated in circumstances whereby testing is not likely to detect all infected birds. It is a costly procedure and not warranted unless there is a definite advantage for the offspring and reasonable assurance that they will not become infected from other sources after delivery to the farm. It has been used successfully for eliminating mycoplasma-infected turkey and chicken breeder flocks.

Destruction of Agent inside the Egg

A pressure differential between the atmosphere and the inside of the egg has been used to force antibiotics through the shell of incubating eggs to prevent transmission of pathogenic *Mycoplasma* species from dam to offspring. This is done by dipping warm eggs into cold antibiotic solutions or using special vacuum machines (2). Antibiotics have also been injected directly into eggs for this purpose (40).

Elevating the egg temperature has also been used to destroy mycoplasmas inside the egg (76). In this procedure, incubator temperature (and internal egg temperature) gradually is raised over a 12–14-hour period to the maximum embryo survival temperature, approximately 46.9°C and then cooled immediately and rapidly to normal incubation temperatures. The procedure usually lowers hatchability.

Treatment of Offspring

Offspring from infected dams may be treated with high levels of antibiotics by injection or feeding or both. This is unreliable, but can be a significant adjunct to other methods and can greatly assist in overcoming economic losses from egg-transmitted diseases that are drug sensitive.

Eggshell-Borne Diseases

Several procedures are used to overcome shell contamination that arises from intestinal contents and other environmental sources. Control involves preventing shell contamination or destroying organisms before they penetrate the shell.

Egg penetration by bacteria occurs more readily if the shell becomes porous. This occurs in the late life of the breeder hen or when there is a deficiency or imbalance between calcium, phosphorus, and vitamin D. Respiratory virus infections can also result in porous and poor shells.

Management of Hatching Eggs

Clean Hatching Eggs

Very dirty eggs should not be used for hatching. If they must be used, they should be dry-cleaned when gathered. The cleaner the shell surface, the less likelihood there will be bacterial contamination and shell penetration.

The most important consideration in hatching egg sanitation is to manage the flock so that eggs are clean when gathered. Many factors enter into accomplishing this goal. Sloping wire-bottom rollaway nests, with or without automatic collecting devices, generally result in clean eggs and a minimum of bacterial contamination.

Clean eggs can also be produced in conventional box-type nests if nesting material is diligently kept clean by continually replacing soiled material. Egg breakage can be reduced by providing sufficient nests for the peak laying period.

The number of floor and yard eggs can be reduced by proper design and location of nests when maturing pullets need them; location and design will vary with the type of house. Nests should be darkened and ventilated, and hens must be prevented from roosting in them at night, because they contaminate the area with fecal deposits.

Keeping the litter dry is an aid in preventing soiled nests and nest material. Proper design and construction of the breeder house to create conditions conducive to keeping litter dry aids disease control at the hatching-egg level. Table-egg breeding stock perform satisfactorily in litterless housing—either all slat or sloping wire-floor houses—and this largely eliminates dirty eggs resulting from tracking litter and feces into the nests. Heavy breeds and turkeys do not perform as well on these floors, so combinations of part slat and part litter are used to aid in litter management.

Measures should be taken to prevent *Salmonella* infections by using *Salmonella*-free feed ingredients, particularly meat meal, eliminating these pathogens from mixed feed (pelleting), keeping feed clean by good feeding practices and storage facilities, and keeping natural carriers (rodents, wild birds, pets) out of pens and houses. Preventing salmonellosis and other types of enteric infections also helps prevent wet droppings, which contribute to wet litter.

Above all, eggs should be gathered frequently, especially in the early part of the day when most hens visit the nests. They should be gathered in clean, dry equipment and held in a dry, dust-free area.

Sanitization of Eggs

The shell surface of hatching eggs should be disinfected immediately after gathering. If sanitization or fumigation cannot be done on the farm, it should be done as soon as possible thereafter, preferably before eggs enter the hatchery building or at the entrance to the egg-processing area. The more delayed the sanitization, the less effective it is because the bacteria will have had longer to penetrate the shell. Unsanitized eggs raise the possibility of carrying a serious infection into the hatchery where susceptible newly hatched chicks are present (see "Disinfectants, Formaldehyde"). Because of possible adverse health considerations resulting from the inhalation of formaldehyde fumes, farm and hatchery personnel should be alert for any new and effective shell sterilization compounds and methods that may become available.

Washing and Liquid Sterilization

Washing eggs with warm detergent solution at a temperature (43–51.8°C) always higher than that of the eggs entering the washing machine—at least 16.6°C higher but not to exceed 54°C—followed by sanitizing the shells with a chlorine compound, quaternary ammonia product, or other sanitizing agent is routine for commercial eggs. The procedure has been employed successfully with hatching eggs, but some real disasters have occurred where thousands of eggs were contaminated rather than sanitized when dirty water was used, especially in recirculating washing machines. Even if eggs are washed properly, very dirty eggs should be cleaned first by sanding to prevent excessive pollution of the washing solution and equipment. If the iron content of the wash water exceeds 5 ppm, it favors multiplication of certain types of bacteria and creates a serious egg spoilage problem. A complete review of egg sanitizing agents is presented by Scott and Swetnam (57).

If egg washing is done, it should be only with a type of machine (brush conveyor type using flow-through wash water principle) that will ensure against contamination with dirty wash or rinse water. Very careful supervision is also necessary to see that all equipment is working properly at all times and is cleaned daily. In some types of machines, if the washing system fails, a few eggs can contaminate the water and, thus, contaminate thousands of others before the problem is detected and corrected. Contaminated eggs in the incubator set off a chain reaction of egg explosions that contaminate surrounding eggs, causing more "exploders" and more contamination. While washing and liquid sterilization of hatching eggs can be done satisfactorily, the procedure is subject to operational difficulties and should not be attempted without full knowledge of the hazards involved.

Whenever cold eggs are moved into a warm, humid atmosphere, moisture condenses on the cold shells (called "sweating"). This moisture provides a medium for the growth of bacteria and fungi already present on dirty or unsanitized shells or originates in contaminated warm air around the eggs. Cold eggs should, therefore, be warmed to room temperature in clean, low humidity air before placing them in an incubator.

Storage Facilities

After fumigation or other shell sterilization, hatching eggs are frequently stored in a cool room (about 10°C) at the hatchery

until set. Cool rooms should be clean and free of mold and bacteria and periodically disinfected to prevent recontamination of shells. Holding hatching eggs too long or under improper storage temperature, humidity, and environment can result in poor quality chicks. Clinical histories indicate that infection in young chicks may sometimes be traceable to fungus-contaminated hatching eggs; infections have been produced experimentally by contaminating shells with fungus spores (75).

Hatchery Management

The building and equipment in which the fertile egg is converted to a day-old chick, poult, or other fowl and the equipment used to process and deliver it to the farm must be clean and sanitary. An individual hatched from a pathogen-free egg will remain pathogen-free only if it hatches in a clean hatcher, is put in a clean box and held in a clean room where it can breathe clean air, and then is hauled to the farm in a clean delivery van.

Design and Location

A hatchery should be located away from sources of poultry pathogens such as poultry farms, processing plants, necropsy laboratories, rendering plants, and feed mills. It is not good practice to retail poultry equipment and supplies from a hatchery building, because this draws producers and service workers who may introduce contaminating material.

A good hatchery design has a one-way traffic flow from the egg-entry room through egg-traying, incubation, hatching, and holding rooms to chick-loading area. The cleanup area and hatch-waste discharge should be off the hatching room, with a separate load-out area. Each hatchery room should be designed for thorough washing and disinfecting. The ventilation system is equally important and must be designed to prevent recirculation of contaminated and dust-laden air. Gentry *et al.* (26) found that hatcheries with poor floor designs and faulty traffic patterns were highly contaminated compared with those with one-way flow.

Importance of Good Sanitation

Factors that aid in obtaining pathogen-free chicks and poults are hatchery cleanliness and sanitation, well-arranged traffic flow, and well-controlled ventilation.

Techniques have been devised for evaluating the sanitary status of commercial hatcheries by culturing fluff samples (74), detecting microbial populations in hatchery air samples (19, 26, 36), and culturing various surfaces in the hatchery (38). By relating results of these techniques to hatchery management, it has been observed by Magwood (37) that bacterial counts of eggshells dropped quickly in clean air, and low counts persisted on all surfaces to completion of hatching. Chute and Barden (18) found fungal flora of hatcheries to be related to management and sanitation programs.

To minimize bacterial contamination of eggs and hatching chicks, hatchery premises must be kept free of reservoirs of contamination, which readily become airborne (37). Trays used for hatching should be thoroughly cleaned with water and then disinfected before eggs are placed in them. This can be done by dipping in a tank of suitable disinfectant (see "Disinfectants"), washing with hot water or steam followed with disinfectant spray, or fumigating with formaldehyde in the hatcher. Trays and eggs are frequently fumigated together immediately after eggs are transferred to the hatcher. Fumigation is sometimes done during the hatch (at about 10% hatch), but concentrations low enough to avoid harming the hatching chick probably serve only to give the down a pleasing yellow color. Formaldehyde fumigation in one case increased the severity of mold infection rather than overcoming it (75). Wright (72) emphasized the practical meaning of hatchery sanitation and how to attain it. He concluded that no fumigation program should be used to replace cleanliness, but rather to supplement it.

As chicks hatch, the exposed embryo fluids collect bacteria from contaminated shells, trays, and ventilating air. The combination of the nutritious fluids and warm temperature forms an excellent environment for bacteria and they multiply very rapidly (26). The cleaner the air and environment to begin with, the more the bacterial buildup is delayed and, as the hatch progresses, the less likely is the navel to become infected (omphalitis).

Breeder Codes

The breeder code is a designation used to denote the source of hatching eggs. It usually denotes breeders of the same age on the same or different farms, all breeders on a particular farm, or any other grouping. There is a tendency to keep breeders in larger flocks and to avoid as much as practicable the mixing of hatching eggs from flocks of many different microbial, nutritional, and genetic backgrounds. If breeders are kept free of disease and fed a good ration, hatching eggs are produced clean and properly disinfected, and chicks are hatched and handled in clean surroundings. Keeping chicks of different breeder codes separated has little practical meaning other than providing that all have more nearly the same level of maternal antibodies against the same diseases. This may permit a more uniform response to vaccines applied to chicks the first 2–3 weeks of life when maternal antibodies have a protective effect.

Occasionally, a disease is believed to be egg transmitted from a breeder flock to the offspring. When this occurs, the disease nearly always appears in several offspring flocks derived from the same breeder flock(s) and delivered to different farms. However, a hatch of chicks is frequently divided into deliveries to several farms and a disease occurs in only those delivered to one farm. This indicates that the disease is farm associated and not hatchery or breeder-flock associated.

Chick Sexers

Unless the output of one hatchery is so great as to demand their full time, chick sexers may go from one hatchery to another, which introduces the possibility of carrying disease. Most sexers are aware of this hazard and are eager to follow proper biosecurity procedures. If sexers must also service other hatcheries, facilities should be provided so that their equipment can remain at the hatchery. They should have a clean area in which to change clothes and wash themselves and their equipment and should

have clean protective garments to wear. Their habits should be at least as clean as those of the hatchery crew.

Surgical Procedures

Poultry can be very cannibalistic under certain circumstances, and beak trimming is commonly practiced in breeder flocks as well as production turkeys and cage layers. In these production systems, beak trimming is a virtual necessity, and special machines have been manufactured for this purpose. Beak trimming is performed on birds of various ages, depending on the husbandry system in use. The extremely dim light used in light-tight poultry houses greatly reduces or prevents cannibalism, but chicks reared under natural or bright light may have their beaks trimmed lightly at 1 day of age or a few days after delivery to the brooder. This early mild trimming is not severe enough to be permanent; therefore, beaks of such flocks of breeders or commercial layers are frequently trimmed again before maturity. Some methods and ages of early beak trimming can protect chickens from cannibalism throughout life if other management factors (e.g., light intensity) are favorable. When this is done properly, there is no serious adverse effect; however, proper beak trimming is more an art than a science, and many birds are permanently handicapped when it is not done properly.

If the operation is done correctly, after the beak tip is removed, the remaining growing tip is cauterized sufficiently with the hot cutter blade to prevent bleeding and regrowth, but not so much that the bird develops a sensitive or abnormal beak that interferes with eating and drinking. Proper beak trimming promotes maximum performance. Done improperly, it is probably the greatest single management cause of unsatisfactory performance of laying and breeding stock. Poor performance resulting from improper beak trimming must not be attributed to some mysterious disease. For more detail on cannibalism and beak trimming, see Chapter 30. Similarly, other surgical procedures, such as removing wattles, combs, or toenails of certain toes, must be done by one trained in the procedure if harm to the bird is to be avoided.

Management Factors in Disease Prevention

The more important physical principles of disease prevention include favorable geographic location of the farm in respect to other poultry units, proper location of buildings in relation to each other and to prevailing wind currents, proper design of the building inside and out, and design and positioning of equipment. Long-range planning and programming of the operation, whether large or small, is very important and should consider movement patterns of various vehicles and equipment, work traffic of regular and holiday caretakers and special work crews, feed delivery and storage, and the system for moving eggs and flocks from the farm. An avian pathologist can be helpful in avoiding some common pitfalls, but to avoid high-risk disease situations, consultation should be done when the farm is being designed and the production programmed, rather than after it is developed and serious trouble is evident.

Good disease-prevention practices are perhaps best illustrated as a chain that is only as strong as its weakest link. Many sound principles can be discredited by failure to carry out one or two related ones, which are either overlooked or not considered essential. Although it may not always be possible to use all the practices, the more that are followed, the greater the chances of avoiding disease outbreaks.

Adult Flocks

Modern laying strains are bred for high egg production, and broiler stocks are bred for rapid growth and good feed conversion. The most important management factor is maintaining feed, water, and environmental conditions at the optimum condition for hen comfort, which in turn results in maximum efficient production and growth. The same is required of meat birds, turkeys, and other types of breeder hens. The egg production or efficiency of feed use will be a good indicator of the success of the management and the welfare of the flock. Many conditions arise that hamper performance, and it is important not only to keep disease out, but to prevent conditions causing discomfort.

Isolation

Not all producers follow the same disease control practices. A close neighbor may disregard sound principles and be burdened with diseases until forced out of business by economic pressures. In the meantime, disease agents present on his premises may be blown or carried by various vectors and fomites to adjacent premises; thus, a disease occasionally may gain entrance even on well-managed units. Until a disease has been eradicated, it serves as a reservoir and potential source of infection for future flocks on the same premises and those on adjacent premises. The closer the houses of one premises to those of another, the more likely is the spread of infection to healthy birds on an adjacent farm.

Some highly concentrated poultry areas have developed because of some favorable condition such as a close market, an available slaughter or processing plant, an accessible feed supply, low-cost land, or favorable climate or zoning. Usually, these areas deteriorate into problem zones of disease of one type or another and resemble huge “megafarms” with many managers, each vaccinating, treating, or exposing birds without regard to the programs of others. Because such areas are in competition for markets, several things may happen. Various advantages may offset disease losses, or the additional cost of production resulting from disease may price the product (meat, eggs) out of the market. In extreme cases, products cannot be marketed either because of the disease or the residues from drugs used to control disease. Producers who do not minimize losses go out of business, and many abandoned poultry farms are purchased or leased by other poultry producers. Some move their operations to a less concentrated area where they usually escape disease, unless they take their problems with them knowingly or inadvertently through carelessness. Those who remain usually upgrade disease-prevention practices by redesigning houses and reprogramming the production cycle. Frequently, reprogramming proceeds to a system of a single age of fowl, permitting complete depopulation at the end of each rearing or laying cycle.

Another solution to area disease problems where farms are too

close even for systematic depopulation to succeed is to develop a coordinated area depopulation and restocking program. All flocks in a reasonably defined geographic area may be marketed at the same time and the houses refilled at the same time. This is more adaptable to broiler production than egg production.

Most serious disease problems could be avoided if a philosophy of premise isolation prevailed from the beginning of an enterprise. No exact minimum distance from other poultry farms can be stated because this is influenced by prevailing winds, climate, type of houses, and other factors. The farther from other poultry farms, the less likelihood of contracting disease from them. Isolation can be effected by taking advantage of segregating space provided by natural or artificial barriers such as bodies of water, hills, cities or towns, or forests, or other interposing agriculture enterprises such as grain, vegetable, or fruit production.

One Age of Fowl per Farm

Removing carriers from a flock and premises is an effective way of preventing a recurrence of some diseases, but it is impossible or impractical for others. The best way to prevent infection from carrier birds is to remove the entire flock from the farm before any new replacements are added and to rear young stock in complete isolation from older recovered birds on a separated farm segment or preferably on another farm and in an isolated area. This practice is often called "all-in, all-out production."

Where birds of different ages exist on a large farm, depopulation seems drastic, but considering mortality, poor performance, and endless drug expense, it could be the most economical solution. Farms and quarantinable farm divisions of up to 100,000 birds of one age prove that size is no deterrent to application of the sound principle of one age of bird per farm or quarantinable segment with programmed depopulation at the end of the production cycle.

Where only one age of bird is maintained, depopulation occurs each time pullets or poults are moved to the layer or breeder premises, each time the broilers or turkeys are moved to slaughter, and each time the old layers or breeders are sent to market. Should a disease occur, the flock can be quarantined, treated, and handled in the best way possible until its disposal. Depopulated premises are then cleaned out, washed, and disinfected, and left idle for as long as possible but at least for 2 weeks before new healthy stock is introduced.

Depopulation is most effective in controlling disease agents that do not survive for long outside the bird. This applies to most respiratory infections (mycoplasma infections, infectious coryza, and laryngotracheitis). It is least effective in controlling disease agents having a resistant state that survives for long periods in nature (intestinal parasites or clostridia).

Started-pullet and pullet-rearing premises are now an established specialized enterprise in the poultry industry. This system has made layer and breeder farm depopulation more practical and successful. As on multiple-age layer farms, serious disease problems may develop and persist on multiple-age rearing farms until they are reprogrammed for a single age or divided into quarantinable, isolated units.



Fig. 1.1. This isolated breeding farm benefits from several fundamental disease prevention and control principles. It is isolated from other poultry farms, is surrounded by forest land, and is divided into quarantinable sections separated by woods as well as distance.

In addition to sanitary practices, environmental factors (temperature, humidity) play an important role in the time interval necessary to prevent carryover of disease. Disease germs begin to die out slowly after elimination from the body. Some (infectious coryza) die out very quickly; others (parasites and coccidia) survive for months or years, depending on whether they develop a resistant stage and on factors discussed in the sections on those individual diseases. In general, the longer a premises remains vacant, the lower the number of surviving pathogens.

Functional Units

For certain economic reasons (breeding farm or small specialized market trade), it is not always possible to limit the entire farm to a single age of poultry. In such instances, it should be divided into separate quarantinable units or areas for different groups of birds (rearing area, pedigree unit, production groups, and experimental birds) (Fig. 1.1). With a suitable arrangement, each area periodically is depopulated, cleaned, and sanitized or can be if necessary. Much stricter security procedures for personnel, bird, and equipment movements are necessary for this type of operation. A very rigid monitoring system is also essential to detect any disease early enough to bring it under control while it is still confined to one quarantinable segment.

No reliable formula exists for minimum distances between houses or units. Windowless and temperature- and ventilation-controlled houses appear to prevent building-to-building and premise-to-premise spread better than open houses. Greater distance can compensate for some inadequacy in building design, human traffic control, and shared equipment. Because each premise and enterprise is different from all others, the poultry producer should seek advice from specialists whose business it is to study diseases and how to prevent and control them.

The most important factor in dividing the farm into segregated units is not so much to facilitate daily separation of farm personnel, equipment, and poultry but to provide quarantinable

units to prevent spread and facilitate elimination of disease, should it occur.

Building Construction

Birdproofing

The first rule in poultry house construction is to exclude free-flying wild birds, because many carry mites and harbor them in their nests. In addition, many species have been found susceptible to some common viral and bacterial diseases of poultry and, thus, could act as carriers. Turkeys on range are especially vulnerable to infections carried by wild birds. For this reason and for generally improved sanitary practices, the trend is to house turkeys, especially breeder and young growing turkeys, in closed or partially closed birdproof houses. Ducks and other domestic waterfowl are also vulnerable to waterborne diseases and to diseases carried by wild birds, especially wild waterfowl and seabirds (gulls, terns, etc.).

Light- and temperature-controlled houses are usually birdproof by reason of their construction (Fig. 1.2). Both ventilation and birdproofing are also achievable in open-type houses in hot climates. Birdproofing is also an important feature of other buildings on the farm (e.g., clean crate and wood shavings, bedding, storage).



Fig. 1.2. Light-, temperature-, and ventilation-controlled houses exclude wild birds and most flying insects. Concrete aprons and paved roadways help prevent tracking of soil-borne diseases into the premises.

Entrances

An apron of concrete at the entrance to a poultry house helps prevent tracking of disease into the unit. Rain and sunshine help keep the apron cleaned and sterilized. A water faucet, boot brush, and covered pan of disinfectant available on the apron for disinfecting footwear are further aids in keeping litter and soil-borne diseases out of the house. Boots must be thoroughly cleaned before the wearer steps into the pan of disinfectant. The disinfectant is useless, however, unless renewed frequently enough to ensure a potent solution at all times.

Ventilation

Poultry buildings should be constructed to provide protection against the elements, yet not create stress conditions such as excess dust, insufficient ventilation with ammonia buildup, excessive draft, damp litter, and situations leading to injuries by mechanical equipment or sharp objects.

Windowless and temperature-controlled houses have many advantages, but one serious drawback has been development in some instances of excessively dry and dusty litter. Although Anderson *et al.* (4) could not demonstrate significant deleterious effects of short-term inhalation of dust by test chickens, it has been observed in practice that colibacillosis outbreaks are frequently associated with inhalation of excessive dust, which must be carried from the building with ventilating air. This may require increased air movement, and precautions must be taken to prevent a stream of incoming cold outside air from blowing directly onto chickens that are prevented, by pen (or cage) arrangement, from seeking shelter.

Coccidial oocysts require moisture to develop into the infective stage. Excessively dry litter inhibits their development and may so limit the number of infective oocysts that infection is too light for a good immune reaction. Conversely, improper ventilation can lead to excessively wet litter, which favors the survival and development of coccidia and other parasites.

Ammonia fumes develop in damp litter and droppings. If ventilation is poor and fumes accumulate, they may reach high enough concentration to inhibit growth and performance, cause keratoconjunctivitis, and exacerbate respiratory infections.

Litter will dry better if it can be stirred frequently, but in spite of all efforts, it may remain wet in winter or in humid climates. If wetness and excess ammonia concentration persist, litter should be replaced and ventilation improved.

Proper ventilation is an engineering science; a good policy is to seek professional advice before installing any system. The influence of such environmental conditions as temperature, humidity, radiation, and atmospheric pollutants on viral disease of poultry has been reviewed by Anderson and Hanson (3).

Floors and Cages

All surfaces inside the building should be of impervious material (such as concrete) to permit thorough washing and disinfection. It is impossible to sterilize a dirt floor!

Raised slatted floors have been used successfully for years for laying chickens, both for adults and for rearing birds. Such floors have alternating wooden pieces and spaces, each about 3/4-in. wide (Fig. 1.3), to permit droppings to fall out of reach of birds and to prevent recycling infection of intestinal parasites and diseases. Because coccidial infection is thus avoided or greatly reduced, poor or no immunity to the parasite develops. This creates no problem for pullets destined for cages or slat-floored laying houses, because immunity to coccidiosis during the laying period in such units would not be an important consideration. If such pullets were transferred to litter-floored laying houses, however, they would very likely become seriously infected with coccidiosis. Commercial meat birds are inclined to develop leg problems and breast blisters if raised on completely slatted or wire floors. A modification of this system, with part of the floor or yard raised slightly and covered with slats, has been used for broiler breeders. The value of this system is increased further by placing



Fig. 1.3. Slat floors aid in the control of intestinal diseases and parasites. Droppings fall through open spaces and out of reach of the flock.



Fig. 1.4. Poultry are kept in cages in well-built, ventilated, light- and temperature-controlled houses in many countries. Good housing reduces stresses associated with variations in weather, and cages reduce intestinal diseases and parasitism.

feed and water over the slatted area, which encourages collection of more droppings out of reach of birds.

Keeping laying hens in some type of cage has become an accepted practice in closed houses (Fig. 1.4) and open-type houses found in hot climates. Cages and wire floors are widely used also to rear pullets destined for cages as adults. The system is so successful in preventing intestinal diseases that birds have no opportunity to develop immunity to them. Coccidiosis is almost certain to occur if chickens or other poultry reared in cages are transferred to litter floors. Drugs can be used successfully to control coccidiosis in these birds, but legal restrictions of their use in meat and egg-producing fowl seriously curtail drug choices for this purpose in laying hens.

Feeders and Waterers

Rats, mice, and other rodents should be kept out of feed because they may introduce and spread salmonellae or other disease agents that can be the source of an outbreak in the poultry flock.

Litter scratched into feed and water troughs and feed spilled in litter increase intake of litter and litter-borne disease agents (e.g., more coccidial oocysts and less coccidiostat are ingested, and a clinical infection may result). If poultry are permitted to consume litter, considerable mortality and depression can occur from impaction of the gizzard, and litter fragments may cause enteritis by mechanical irritation.

Feed troughs should have some type of guard to keep poultry out and should not be overfilled so that feed is spilled into litter. Feeders without guards permit defecation into feed, which encourages spread of diseases shed in feces. Wet feed in litter provides a good medium for growth of molds, which can cause liver, kidney, immune system, and other damage to the well-being of poultry. Growing and laying cages for egg production flocks in light- and temperature-controlled houses eliminate most of the problems associated with litter. Many good automated feeding and watering systems are available commercially, but sometimes these are not installed or oriented as the manufacturer intended, and consequently health problems develop.

Roost areas over screened dropping pits are common in floor-laying and breeder hen houses to keep chickens away from their feces. Screened roost areas are also desirable in rearing houses for layers and breeders to prevent piling by the birds and excessive fouling of litter with feces, which in turn leads to packing and caking. Feeders and waterers over the pits keep the birds on the roost area much of the daytime as well as at night, so most droppings collect out of reach. Spilled water also falls under the roosts, so the litter area stays drier.

Waterers are frequently set or hung over the litter area. In this case, waterers should be managed so that spillage onto the litter is minimized. Waterers can be put into two basic categories: those that provide a constant reservoir of water, which is maintained automatically (troughs, cups, and hanging plastic bells), and nipple drinkers (Fig. 1.5), which supply water on demand when activated by a bird. Waterers that provide an open reservoir of water must be cleaned and disinfected regularly to prevent the buildup of potentially pathogenic organisms in the water supply. These waterers are also more prone to spillage and the associated problems of wet litter. Starting day-old birds is somewhat easier with waterers that have an open and visible water reservoir. The advantages of nipple drinkers are found in the significant im-



Fig. 1.5. Nipple drinkers are effective in preventing microbiological contamination of clean water and help maintain dry litter conditions.

provement they offer in providing water free of organisms commonly found in the poultry house environment and in decreased water spillage. The drier litter conditions afforded by nipple drinkers result in decreased multiplication or maturation of coccidia, bacteria, and fungi in the litter. Broiler production managers report a decreased incidence of infectious disease with the conversion of poultry buildings to nipple drinkers. Nipple drinkers have been developed that are suitable for most types of poultry production.

Feed and Water Medication

In spite of all precautions, poultry may become sick. This should be recognized from the start, and facilities for quick treatment by medication in water or feed should be provided long before it is needed. When birds are grouped by tens of thousands in one big pen, segregation and treatment of individuals is impractical; mass medication and vaccination are essential if any treatment is to be given.

Feed medication is not the best method of treatment because of the inappetence of sick birds and their inability to compete for feed. Water medication is better because the sick will frequently drink when they will not eat. Mass medication, while not completely successful in curing the sick, may hold the disease in check until the host can respond with a successful immune response. Provision should also be made for mass vaccination through drinking water, as this is an accepted and successful labor-saving practice. If drinking water is chlorinated or otherwise treated, the sanitizing agent may destroy the vaccine, so provision must be made to permit the use of untreated or distilled water for mixing and administering water vaccines.

Several methods can be used to reduce, remove, or neutralize chlorine in chlorinated water supplies. The only practical method for dealing with this problem on poultry farms is to add protein to the water when mixing water vaccines. A common practice is to add 1 cup of nonfat dried milk to 50 gallons water in tanks or canned liquid nonfat milk mixed with vaccine in a proportioner.

If a building is constructed with a bulk water tank for gravity-flow watering devices, the tank should be of plastic or lined with some nonreactive protective substance and be readily accessible for cleaning and for mixing medicaments. If the watering devices are operated on high pressure, the pipe leading into the pen should have a bypass system with proper valve arrangement so that a medicament proportioner can be installed quickly when needed. A metering device to measure feed and water consumption is useful to keep track of the health of the flock. Float-regulated or continuous-flow water troughs can spread disease within a house. Infectious coryza has been observed to spread down cage rows of chickens in the direction of water flow. Use of a watering system with nipple drinkers for individual cage units will aid in preventing spread of disease.

Bulk feed delivery, metal bulk storage tanks, and automatic feeders are common in modern poultry operations. These eliminate the possibility of rodent contamination, because feed is always in closed tanks rather than in bags or open bins, but the system leads to difficulties when short-term emergency medication in feed is desirable and the bulk tank is full. Two alternative systems are useful: An additional smaller bulk tank may be installed just for emergency medicated feed, or a small dispensing tank may be interposed between the bulk tank and feed troughs so that emergency medicated feed can be put in the smaller tank by hand.

Flock Placement and Management

Handling the Young

Chicks and poults hatch with a reserve food supply in unabsorbed yolk sufficient to sustain them for about 72 hours. Some offspring actually hatch 1 or 2 days before they are taken from the hatcher; therefore, they should receive feed and water as soon as possible, preferably within 24 hours after removal.

Brooder Temperature

Chilling, overheating, starvation, and dehydration are serious stress producers and can precipitate active disease from latent infections that might otherwise be overcome by the young without detectable symptoms. In a randomly split hatch of chicks from the same group of dams, those delivered to one farm can suffer much greater mortality than those delivered to others. This is associated with differences in environmental stresses and exposure to disease. Young chicks and poults should be kept at a comfortable temperature at all times. The brooder temperature is usually started at 35°C and gradually is reduced as the birds mature. Although thermometers are helpful, strict adherence to thermometer temperatures without regard to obvious discomfort of chicks or poults is poor practice. An uncomfortable bird lets the caretaker know about it. Its peeping should be heeded, and the cause of discomfort corrected, regardless of thermometer reading.

Coccidiostats
Floor-reared poultry receive coccidiostatic drugs in feed from the first day to prevent coccidiosis (broilers, turkeys, replacement pullets destined for cage adult housing) or to keep the disease under control until birds develop active immunity (breeder flocks, replacement pullets destined for floor adult housing).

Immunity, however, depends on a number of factors. The amount of feed and coccidiostat intake may vary among birds, and the number of viable sporulated oocysts will vary with differing humidity, temperature, and litter conditions, even in different areas of large buildings. Depending on the relationship of these variable factors, the coccidial infection may be too mild to elicit good immunity, or it may be so severe that a frank outbreak occurs. There is no special management formula to overcome this dilemma other than a keen awareness of the variable factors and an attempt to maintain the proper physical environment to favor the degree of infection desired (see Chapter 28).

Feed and Water Consumption and Medication
Scientific feed formulation is the business of highly trained nutrition experts, and quality feeds are the rule, not the exception. Poultry eat the feed, not the formula, however, and occasionally problems arise that are traceable to feed (accidental omission of an ingredient, low-potency vitamin supplement, moldy or toxic contamination of an ingredient).

More important in everyday disease control are variations in feed consumption associated with hot or cold weather; housing changes; breed, type, strain, and age of bird; body weight; rate of lay; energy and fiber content of feed; and particle size of feed ingredients. With a 10–20% lower feed consumption associated with one of these factors, there is also a lower intake of coccidiostat or other medicament in feed by the same amount. Conversely, an increase in total feed consumption as a result of one of these factors increases total intake of all feed ingredients, including drugs.

Increased water intake during hot weather can spell disaster through overconsumption of water medication, but a given concentration of a drug in water may fail to control a disease under circumstances in which consumption is very low, as in very cold weather. Also, if natural sources of water are available the intake by some birds from the trough may be light. Many are the tragedies from overdosing due to carelessness; miscalculation; or failure to consider feed and water intake, weather, and other variables. When drugs are used in feed, great care should be exercised in adding the same or other drugs to water.

Biosecurity
Biosecurity provides safety from transmissible infectious diseases, parasites, and pests-is a term that embodies all of the measures that can or should be taken to prevent viruses, bacteria, fungi, protozoa, parasites, insects, rodents, and wild birds from entering or surviving and infecting or endangering the well-being of the poultry flock.

The reader is referred to *Biosecurity for the Birds,* a videotape (or DVD) that illustrates biosecurity measures and the many threats to poultry health that biosecurity is designed to prevent. This video was produced by the United States Department of Agriculture (USDA), Animal Plant Health Inspection Service (APHIS), Veterinary Services. Inquire at your state APHIS veterinary office for further information. This professionally filmed material is also available from state extension offices, state health officials, online, and major poultry industry trade associations. It provides clear biosecurity training for workers, managers, and owners of all types of birds.

Biosecurity Guidelines
Specific disease-prevention guidelines, targeted to different sectors of industry (truckers, service workers, farm owners, catching crews, and others), are often available from university poultry extension specialists, and many of these are available online.

Sources of Infection and Protective Measures Against Them

Infections gain entrance to a flock from various sources. To understand why various preventive practices are recommended, it is important to review briefly the sources and routes of infection.

Humans
Because of their mobility, duties, curiosity, ignorance, indifference, carelessness, or total concentration on current profit margin, humans constitute one of the greatest potential causes of the introduction of disease. Rarely is this because they become infected and shed the disease agent, but rather because they track infectious diseases, use contaminated equipment, or manage their flocks in such a way that spread of disease is inevitable.

Most frequently, footwear is suspected as the means of disease transport, but hands can become contaminated with exudates when lesions and discharges are examined. Clothing can also become contaminated with dust, feathers, and excrement. At least one avian disease pathogen (Newcastle disease virus) has been found to survive for several days on the mucous membrane of the human respiratory tract and has been isolated from sputum (68).

The backyard flock maintained without regard for disease control can perpetuate a disease that constitutes a threat to a large productive industry. However, because most such flocks are not vaccinated, they may be susceptible to diseases against which large commercial flocks are protected. The greatest hazard to commercial producers that is created by fancy breeds and backyard flocks is the possible perpetuation of diseases that have been eradicated from the industry. Thus, it is a sound principle of disease prevention that no employee of a commercial unit have any contact with poultry, pet, or hobby birds, at home or elsewhere.

Neighbors
A frequent source of infection is a disease outbreak at a neighboring farm. Disease inspection visits among producers are a common way of spreading disease. If a neighbor's flock is afflicted with a very interesting new disease, discuss it by telephone. It is best to warn neighbors not to visit when a disease is

in progress, and by all means, do not walk around on a neighbor's farm for any purpose.

Contract Work Crews

Much of poultry farm procedure requires sporadic use of a crew of several workers (e.g., blood testing, beak trimming, vaccinating, inseminating, sexing, weighing, and moving birds from one location to another). The producer or farm manager frequently has difficulty in assembling a crew who are available and knowledgeable about handling poultry. Therefore, crews who service many poultry enterprises are contracted. Such crews travel about the poultry community handling many flocks and must be regarded as a potential source of infection. Thus, they should take stringent precautions to safeguard the health of every flock with which they work.

Visitors

Disease outbreaks in a community have been known to follow the path of a careless visitor. If visitors do not enter premises or buildings, they cannot track in diseases.

The source of a new or dreaded disease is often puzzling. World trade and travel are becoming more commonplace. It is not uncommon for a person to leave one farm in the morning and be visiting another farm or place of business in another part of the country or another continent on the same day. Some disease agents can survive that time frame easily. All who travel should be cognizant of this and guard against introduction of disease into their own flocks or onto the premises of clients, competitors, friends, or fellow producers when returning from a trip. Protective footwear and clothing are not readily available in all countries and poultry areas. A good preventive measure when returning from a trip is to sanitize shoes and launder all clothing worn on farms.

Recovered Carriers

Carrier birds are those that have apparently recovered from a clinical infection but still retain the infectious organism in some part of the body. Although they appear healthy, the infectious agent continues to multiply in the body and to be eliminated into the environment. Like actively infected flocks, they can perpetuate a disease on a farm and constitute a disease threat to other birds. Many commonly occurring diseases are known to be transmitted by carriers. Carrier birds can be a potential source of disease through the various practices noted in the following sections.

Multiple Ages

Multiple ages on a premises constitute a serious disease potential from both actively infected fowl and recovered carriers, particularly if birds of differing ages are closely associated through management practices or proximity. Disease agents that result in chronic infections or recovered carriers are passed by various means, including direct contact, to each new susceptible flock brought onto the premises. Serious drops in production may occur in young laying flocks moved onto laying premises where carrier birds from previous disease outbreaks remain. Whenever possible poultry producers should practice all-in and all-out production.

Started Pullets

Pullets frequently are reared to or near point-of-lay by a specialized pullet rearer or on a separate premises unit belonging to the laying-farm owner. This practice has become established in the industry for many sound reasons. Pullets can, however, be a potential source for introduction of a disease onto a layer farm if they have been exposed on the pullet farm and, as a result, have become recovered carriers of some disease not existing on the layer farm. Another hazard is assembling mature pullets reared in different geographic areas onto a single layer premises, even an all-in, all-out layer premises. Those reared in one area may have been exposed to and recovered from, but carrying, a disease agent not found in the area where the other pullets were reared.

Induced-molted Hens

Induced molting of laying hens or breeders is frequently practiced (particularly during times of economic stress) to supply a special market, meet an emergency egg demand, or improve declining shell quality, or because it is deemed economical at the time. One advantage of keeping induced-molted hens, rather than rearing new replacement pullets, is that old hens are not apt to suffer a disease that normally occurs during the rearing age. If such flocks are molted and held in the same house, there is little danger of disease problems developing. Conversely, a producer who collects spent hens for molting from many poultry farms and mixes them on one premises at one time runs a serious risk, because any of the molted groups may be carriers of a disease to which the others are susceptible.

Poultry Show Stock

Birds exhibited at poultry shows may be exposed to actively infected or symptomless carrier birds of other exhibitors from which they may contract disease. The contact-infected stock may not develop active signs until returned to the owner's farm, where they may then be a source of new infection. Breeders of fancy birds, game birds, and youths with poultry projects (4-H, Future Farmers of America) must recognize the extreme hazards of returning birds exhibited in shows and fairs and of introducing partly grown or adult birds for special breeding purposes. A cardinal rule for show stock is that it should never be returned to the owner's farm. If birds must be shown, individuals should be selected that can be sold after the exhibition. If they must be returned, they should be quarantined for several weeks. In some areas, exhibition-type poultry should be vaccinated against some diseases. A local avian pathologist or other poultry specialist can often provide appropriate vaccine schedule information.

Breeding Stock

Adult stock considered especially desirable for breeding purposes may be symptomless carriers and serve as a source of infection for the breeding farm. It is best to purchase such stock as hatching eggs or day-old chicks and to rear them in an isolated

off-farm quarantine area until there is reasonable assurance that they are free of infection.

Mixed Species of Poultry

One species that is naturally very resistant to a disease may act as a carrier of that disease for another species that is very susceptible. Some death losses and debilitation from histomoniasis may occur in chickens, but in turkeys, the losses can be disastrous. Therefore, even with the routine use of drugs to prevent histomoniasis, the two species should never be raised together, and turkeys should not be reared in a facility that has recently had chickens on it.

Also, a silent (inapparent) mycoplasma infection in chickens may spread to mycoplasma-free turkeys and erupt into a full-blown case of sinusitis and air sac infection. Other diseases may be rather innocuous in one species of fowl but very serious in another. It is also advisable to keep meat and laying chickens separated, because the same disease may have different economic importance in the two types.

Other Sources

Hospital Pen

Sick birds from several pens collected into one hospital pen or house and later returned to their respective quarters may carry back not only the condition for which they were removed, but one or more diseases contracted while in the hospital area. Therefore, hospital pens are not recommended for routine segregation of sick birds, except as a way-station en route to the diagnostic laboratory or crematorium. If and when used for a special purpose (observation, injury, or cannibalism), they should be temporary arrangements within the house and should hold birds from only one pen or house.

Backyard and Pet Fowl

Poultry kept as pets or to supply household eggs or meat are just as capable of carrying and transmitting disease as are commercial flocks. Pet barnyard fowl of a rare or interesting nature may also carry disease to commercial poultry. The risk to the invested enterprise is too great to permit such a part-time hobby by a resident owner or employee. Cockfighting is banned in many states, but these game fowl are commonly transported around the country, constituting an effective way to carry disease. Some employees may own or handle these fowl and, thus, could introduce a serious disease to the poultry enterprise where they work. Poultry farm and hatchery owners and workers should be especially cautious about contact with imported ornamental pet birds or migratory waterfowl because they can be symptomless carriers of diseases that are highly virulent for domestic poultry.

Live-Bird Markets

These are buildings usually in the inner cities in which poultry of all types, ages, and health status are assembled by small buyers to supply a demand for live fowl for those who wish to examine the fowl live prior to slaughter or prefer to kill and dress fowl at home (Fig. 1.6). Such facilities are rarely depopulated, cleaned, and disinfected, and thus are ideal situations for transmission and propagation of poultry diseases. In addition, the hauling equipment and vehicles may not be cleaned and disinfected after each use. Such equipment, hauled throughout the poultry industry areas where a few birds of different types or age are bought at various places, is an excellent means of transmitting diseases. Good managers and owners will keep such buyers and their equipment out of their farms and offices. The live-bird market trade has been strongly associated with the propagation and spread of avian influenza and laryngotracheitis.



Fig. 1.6. Urban live-bird markets are rarely depopulated and disinfected. Diseases are propagated readily and transmitted to new birds brought in to replenish the supply. Commercial production managers are sometimes tempted to deal with live-bird markets because of the increased profits afforded by this association. These short-term profits are extremely small compared to the severe losses that can occur if infectious agents of high pathogenicity are introduced into the commercial poultry industry. (University of Maryland)

Egg-Borne Diseases

Egg-borne diseases are transmitted from the infected dam to newly hatched offspring by means of the fertile egg. Some disease agents are carried inside the shell as a result of shedding into the egg prior to the addition of the shell and membranes. Others are carried on the shell or penetrate from the shell surface through natural pores after the egg is laid.

The agent may gain entrance to the egg as a result of infection of the ovary and ovarian follicles (transovarian transmission), as a result of contamination of the free ovum in the peritoneal cavity, or by contact in the oviduct. After the shell and membranes are added, the organism enjoys a protected location where it is not easily destroyed. From there, it can later invade the developing embryo, and lesions are frequently observed in tissues and organs of offspring at hatching. Transovarian transmission seems to be limited to only a few of the many diseases that affect poultry, and most of these have been eradicated from breeding flocks.

When the freshly laid egg cools from body temperature to nest, room, or cool-room temperature, a pressure differential occurs between the inside of the egg and the atmosphere. Any fluid on the shell surface is drawn inward. Motile bacteria are aided by this pressure differential to penetrate the shell. The primary contami-

nation of this nature is from enteric organisms, particularly salmonellae and coliforms, but other types of bacteria and fungi also may be drawn into the egg. For preventive measures, see "Breeder Flock Management" and "Management of Hatching Eggs."

Equipment

Diseases and parasites can be carried on equipment. Cleaning equipment and vehicles usually have accumulations of litter and feces that can be a threat to other farms and houses where they may be transported for succeeding assignments. They should be washed free of litter and droppings before use in another farm area. Types of vehicular traffic that must be carefully controlled are feed trucks, live-haul trucks, vehicles used in dead bird disposal, and manure spreading equipment.

Mites frequently are found on eggs and can be transported from farm to farm in corrugations of egg cases taken into chicken houses. Wire crates and baskets do not offer these hiding places. Residues of *Salmonella*-contaminated eggs on egg flats may be a potential method of introducing disease. Use of washed and disinfected plastic egg flats and moving of stacked flats of uncased eggs on racks and pallets reduce the hazard of transmission of diseases and parasites among farms.

Fowl pox, infectious bursal disease, and Marek's disease viruses, coccidia, roundworm eggs, and other infectious material can be carried on crates, footwear, and vehicles, particularly on the floor and foot control pedals of a vehicle.

Artificial inseminating equipment, particularly reused inseminating tubes, offer an excellent method of transmitting disease.

Poultry hauling equipment can disseminate infectious material through feathers, feces, blood, exudates, and skin encrustations left in the crates or picked up at the slaughter plant. Hauling equipment should be washed and disinfected after use before being taken to another farm (Fig. 1.7).



Fig. 1.7. Soiled vehicles and equipment can carry disease agents. They should be thoroughly washed and disinfected after each live haul. One gram of chicken manure can contain enough viral particles to infect 1 million birds with avian influenza.

Miscellaneous Sources

Laboratory Exposure

Frequently, a producer, particularly a small flock owner, hobbyist, or game bird owner, will want to take a bird home after the veterinarian has examined it at the laboratory. While in the receiving area or diagnostic facilities, even for a short time, live poultry have a good opportunity to contact some disease agent. Except under special circumstances (exotic birds, valuable pet), no bird should be returned from the laboratory to the farm, because it could develop disease and be the source of a new infection on the home premises. The bird should be either sacrificed and necropsied or, if a pet, referred to a private veterinary practitioner.

A disease may be tracked from laboratory surroundings to a farm by careless laboratory or service workers or the producers. Clean and frequently washed and disinfected laboratory areas are the responsibility of the veterinarian. Precautions against tracking disease from the laboratory to the farm are the responsibility of the producer and service worker.

Rodents

Rodents contaminate feed and litter with their excrement. They are particularly hazardous to *Salmonella* control, because they are frequently infected with these organisms and can perpetuate the disease on a farm.

Household Pets

Dogs and cats, like rodents, are capable of harboring enteric organisms that are infectious to poultry. When these pets are not confined to the household area, but roam continually among the poultry in the pens and yards, they constitute a serious health hazard. Such pets are just as capable of tracking contaminated material on their feet and in their hair as people.

Wild Birds

Wild birds are capable of carrying a variety of diseases and parasites. Some cause infection or illness in the wild birds themselves; for others, the birds act as mechanical carriers. Every effort should be made to prevent their nesting in the poultry area. Imported zoological specimens destined for zoos are not a direct contact threat because the zoos are located in cities, but they should be considered as a potential source of introduction of an exotic disease or parasite. Exotic ornamental pet birds constitute a real hazard because they become widely dispersed and may be purchased by poultry workers. On numerous occasions, exotic birds in or destined for pet stores have been found infected with a virulent exotic form of Newcastle disease virus, which in at least one instance was the source of a serious and costly outbreak in poultry. Stringent entry quarantine requirements to apprehend and destroy infected birds provide a good barrier against the introduction and dissemination by carrier birds, but failures can occur (illegal smuggling), and producers should be wary of such personal pets. Domestic pigeons can also be a source of dangerous strains of Newcastle disease virus.

Insects

Many insects act as transmitters of disease. Some are intermediate hosts for blood or intestinal parasites; others are mechanical

carriers of disease through their biting parts. Still others, because of their feeding habits and hiding places, appear to be reservoirs of disease, whereby the infectious agent survives from one flock to the next.

Feed

Some ingredients may contain infectious agents, particularly salmonellae, from contamination at their source or anywhere along the production line or storage areas. Methods are available for sterilizing feed, but they increase the cost of the final product. Pelleting, if done properly, is a practical method of greatly reducing contaminants because of the heat generated in the process, but it is not dependable for complete sterilization. Meat meal is the feed ingredient most apt to introduce *Salmonella* spp. This hazard can be avoided by using only vegetable protein ingredients, supplemented as necessary with synthetic amino acids. Such formulations are recommended for breeder rations if pelleting capabilities are not available.

Personnel Control

Company and Farm Personnel

Managers, supervisors, and owners are sometimes the worst offenders at breaking biosecurity rules. These people frequently visit many different types of poultry enterprises, farms, and farm units, and disease agents do not respect authority or ownership. Such personnel, like veterinarians, should set a good example for the workers. One of the most important aspects of disease control is an awareness on the part of everyone—owner; workforce; feed and supply delivery people; egg, bird, and litter haulers; and all who visit or work on poultry farms—that each has an important role in the disease-prevention program. Assembling the workforce for occasional educational conferences on health goals and reasons for procedures will foster awareness. This is as important as the preventive measure established. It is also a good occasion to use the biosecurity video mentioned previously in this chapter.

In designing buildings and farm layout and in programming production and management, it is important to make every disease-preventive practice as easy and efficient as possible. Any procedures that are difficult probably will be done incorrectly.

Visitors and Customers

For some types of poultry enterprise, it is deemed necessary to show the birds, premises, or procedures to visitors. In such cases, an observation booth, platform, or fenced area should be provided. Such an area should be sealed off from the poultry pens or hatchery. For maximum security, the entry, access road, and observation area should be completely separated from the work area.

Visitors can be a minimum hazard if proper provisions are made to accommodate them and they cooperate fully with strict sanitary rules. When visitors must enter the poultry quarters, it is most important that they wear disinfected rubber overshoes and other footwear; in addition, they should wear protective clothing such as clean, laundered, or new disposable coveralls and hat. Disposable plastic boots may become punctured when used on gravel or other sharp surfaces; therefore, only heavy gauge (5 mil or greater) plastic disposable boots should be used. These sanitary precautions are most essential when entering floor brooding and rearing houses but will help keep disease out of any facility.

Vaccination

Purpose of Vaccination

Vaccines in poultry production are used to prevent or reduce problems that can occur from infection of a field strain of a disease organism. Vaccines and vaccine programs vary widely in their effectiveness, and this is frequently by design. Some vaccines are designed to incite high levels of immunity to protect birds in the face of aggressive endemic disease challenges, such as viscerotropic, velogenic Newcastle disease. These vaccines may cause a mild form of the disease themselves but are deemed appropriate and useful because of the risk associated with eventual infection of the deadly field pathogen. Vaccine selection and how they are programmed frequently becomes an exercise in risk management and cost efficiency. Local conditions must always be considered when evaluating and critiquing a vaccination program.

A second reason for the vaccination of poultry flocks is to hyper-immunize hens to maximize maternally derived antibody passed through the egg to the hatching progeny. Chicks frequently receive up to 3 weeks of protection from maternal antibodies allowing their immune system to mature to a level capable of eliciting an efficient active immune response if exposed to a potentially harmful virus or bacteria. Antibodies are not always completely protective but for viruses such as infectious bursal disease (IBD), many areas of the world have found maternal antibodies a very useful tool in IBD prevention and control.

Types of Vaccines

Poultry vaccines are typically characterized as live or inactivated. General characteristics of vaccines are summarized in Table 1.1 (14). Live vaccines are available for numerous viral, bacterial, and coccidial organisms.

Techniques used in the development of live vaccines have varied widely. Table 1.2 shows some of the most common methods used to generate an acceptable live vaccine candidate and examples of each method.

Live vaccines are widely used throughout the world because they are commonly effective when mass applied, and they are relatively economical. Immunity from live vaccines is generally short-lived, particularly following initial exposure. Some exceptions to this exist for vaccines such as laryngotracheitis, fowl pox, and Marek's disease.

For live vaccines to work as they were designed, they must be stored, mixed, dosed, and applied appropriately. Storage of live vaccines is generally in a dark, refrigerated area. Liquid nitrogen freezing of live vaccines preserves and prolongs cell culture viability that is essential for cell-associated vaccines such as Marek's disease vaccines. Licensed live vaccines have an expiration date printed on the vial that, if stored according to label directions, ensures that the appropriate minimum dose is maintained through the dating period. Shelf life varies widely on live vaccines but most generally are licensed with 18 months to 2 years dating.

Table 1.1. General characteristics of live and inactivated vaccines for poultry.

Live vaccines	Inactivated vaccines
Smaller quantity of antigen. Vaccination response relies on multiplication within the bird.	Large amount of antigen. No multiplication after administration.
Can be mass administered—drinking water, spray.	Almost always injected.
Adjuvanting live vaccines is not common.	Adjuvanting killed vaccines is frequently necessary.
Susceptible to existing antibody present in bird.	More capable of eliciting an immune response in the face of existing antibody.
In immune bird, booster vaccination is ineffective.	In immune bird, additional immune response frequently seen.
Local immunity stimulated (i.e., trachea or gut).	Local immunity may be re-stimulated if used as a booster but poor if not a secondary response.
Danger of vaccine contamination (e.g., egg drop syndrome, reticuloendotheliosis virus).	Little danger of vaccine contamination.
Tissue reaction commonly referred to as a "vaccine reaction" is possible and frequently visible in a variety of tissues.	No microbe replication; therefore, no tissue reaction outside that which is adjuvant dependent.
Relatively limited combinations—due to interference of multiple microbes given at the same time (e.g., infectious bronchitis, Newcastle disease virus, and laryngotracheitis).	Combinations are less likely to interfere.
Rapid onset of immunity.	Generally slower onset of immunity.

Table 1.2. Methods of generating live vaccine candidate.

Method	Example
Virulent organism inoculated to a less susceptible target tissue or at a controlled dose	Laryngotracheitis-cloacal route
Naturally occurring mild pathotype	*Mycoplasma gallisepticum* F strain
Egg passage of virulent parent	Infectious bronchitis—Arkansas strain
Temperature-sensitive mutant of virulent parent	Turkey coryza vaccine—*Bordetella avium*
Chemically derived mutants of virulent parent	M-9 Fowl cholera vaccine
Tissue culture / passage of virulent parent	Laryngotracheitis
Combination of egg passage and tissue culture passage of virulent parent	Infectious bursal disease—Lukert virus
Plaque selected "clones" of parent virus	Newcastle disease virus—cloned Lasota vaccines
Selection of subpopulations or organisms based on replication characteristics *in vivo*	Precocious strains of *Eimeria* spp.
Relatively virulent organisms given at an age that minimizes disease	Avian Encephalomyelitis

Mixing directions also vary widely, but many recommend the use of a water stabilizer such as powdered skim milk. Water stabilizers minimize some of the negative effects of residual chlorine, metals, and high temperature on the reconstituted virus. Cell-associated Marek's vaccines generally have very specific diluents aimed at maintaining cell culture viability through the time period between reconstitution and inoculation. The dose needed to get an appropriate immune response from a live vaccine is frequently dependent on the virus, genetic background of the bird, age of the bird, existing circulating antibody within the bird, and the method to be used when applying a vaccine. Vaccines generally are licensed based on protection studies performed in an SPF-type leghorn bird, absent of any circulating antibody to that particular agent, at the youngest age on the label, and at the minimum titer expected at the end of the dating period allowed for each given vaccine. With all these variables, it is not hard to imagine why clinical veterinarians and other health professionals may adjust dosages of live vaccines according to local field conditions.

Severe vaccine reactions or insufficient protection can result from misjudging any of these variables. As a final note, poultry house conditions and local disease risks need to be taken into account when optimizing the use of live vaccines.

A second type of live vaccine is emerging with the development of genetically engineered, live virus and bacteria vectored vaccines and gene deletion mutants of a pathogenic parent organism. The recombinant vaccines are made using live virus or bacteria, as a vector to transport the gene coding for the protective antigen of a second infectious agent, for which immunity is desired. Examples of live virus-vectored vaccines include recombinant fowl pox virus vaccine expressing genes to protect against H_5N_2 avian influenza (9), fowl pox virus expressing Newcastle disease virus antigen (13), fowl pox virus expressing infectious bursal disease virus (7), and baculovirus expressing infectious bursal disease virus (69). Bacteria-vectored vaccines described in poultry include bacteria such as *E. coli* (32) and *Salmonella* spp. (54) expressing antigens from coccidia and *E. coli*, respectively. A vaccine to reduce salmo-

nella infection, made from a gene deletion mutant of *Salmonella typhimurium* (20), is commercially available.

These recombinant and gene deletion mutant vaccines have been shown to be relatively protective, when compared to controls, against pathogenic challenge under experimental conditions. The efficacy and cost effectiveness of the recombinant vaccines under field conditions are yet to be determined. This type of vaccine may offer advantages where the spread of traditional vaccines to susceptible populations cannot be properly managed. Additionally, these technologies allow for diagnostic differentiation of vaccine from virulent field challenge. This property may be useful when utilized in eradication programs such as laryngotracheitis. Regulatory considerations when acquiring a federal license for vectored vaccines include demonstrating the genetic and phenotypic stability of recombinant viruses or bacteria and documenting any alterations in the host range or tissue tropism of the recombinant organism, as compared to the parent organism (41).

Inactivated vaccines or killed vaccines used in poultry are generally whole bacteria or virus preparations combined with an adjuvant that are designed for subcutaneous or intramuscular injection. They are frequently, but not always, used in commercial egg layer and breeding birds to stimulate long-lasting immunity and/or antibody levels to specific antigens. Inactivated vaccines generally consist of two distinct components, often referred to as aqueous and adjuvant phases, emulsified into a homologous liquid. The aqueous phase contains the antigen, and the adjuvant generally enhances the bird's response to this antigen. The ratio of antigen to adjuvant differs greatly depending on the vaccine. This ratio generally is determined by factoring in the properties of the adjuvant(s), the antigen(s), viscosity, immune response, and tissue reactivity. Mineral oil is the most commonly used adjuvant, although aluminum hydroxide is a common alternative in notoriously reactive inactivated vaccines such as fowl cholera and infectious coryza. Adjuvant technology continues to grow, and vegetable, fish, and animal oils used as adjuvants offer some opportunities for lower viscosity, immunogenic vaccines (63). Injection of humans that are administering these inactivated vaccines should be avoided. Serious injuries have been reported from accidentally injecting vaccine into a finger or hand. The site of injection can become swollen, red, and painful, and the function of the area may be affected. Victims should seek medical treatment at once and inform attending physicians of the organism(s) and adjuvant contained in the inactivated vaccine.

DNA vaccines are an entirely new type of vaccine that has evolved in the late 1990s. These vaccines can achieve both humoral and cell-mediated immunity, are similar to live vaccines, and have the relative safety associated with inactivated or vectored vaccines. DNA vaccines have been used successfully in poultry for avian influenza and Newcastle disease in chickens (24, 56) and duck hepatitis B in ducks (67). Although promising, DNA vaccines have both technological and economical challenges to overcome before they are commercially viable.

Vaccine Delivery Systems

Improper vaccine application is the most common reason vaccines and vaccine programs fail. With the success and growth of the poultry industry throughout the world came tremendous challenges in efficient and economic application of poultry vaccines. The most commonly used application techniques in commercial poultry include *in ovo* at 17–19 days of embryonation, subcutaneous or intramuscular injection at day of hatch, spray in the hatchery, intraocular or nasal drop in the hatchery or on the farm, spray on the farm, through the drinking water on the farm, wing web stab, and subcutaneous or intramuscular injection on the farm.

In Ovo Vaccination

In ovo vaccination is performed during the process of transferring incubating eggs in the hatchery from the setter to the hatcher. After poking a hole in the shell, vaccine, most frequently Marek's disease vaccine, is injected just under the membranes at the floor of the air cell. Depending on the embryo age at transfer, generally between 17 and 19 days of incubation, approximately 25–75% of the vaccine (0.05 ml in most cases) is injected into the area of the neck and shoulder. In the remaining 25–75%, vaccine is administered into the extra embryonic compartment (27). The original experiments on *in ovo* vaccination with Marek's disease vaccine showed that chicks were protected earlier than those vaccinated after hatch (58). However, in the United States, where more than 80% of broiler chickens are vaccinated against Marek's disease *in ovo*, the primary reason for its acceptance has been the labor savings when compared to day-old injection (66). Using an egg injection system (Embrex Inovoject® Egg Injection System, Research Triangle Park, NC), one machine with three people generally inoculates 20,000–30,000 eggs per hour (Fig. 1.8). This method of vaccination leaves a hole in the egg for the final few days prior to hatch and in poorly sanitized hatcheries has resulted in poor early livability due to bacterial or fungal contamination while in the hatcher. Hatcheries must be acutely aware of their aspergillosis levels to run an egg injection system successfully (71).

Subcutaneous or Intramuscular Injection at Day of Hatch

Day-old vaccination, most commonly using Marek's disease vaccine, is generally accomplished by giving 0.2 ml of vaccine subcutaneously under the skin at the back of the neck or 0.5 ml intramuscularly in the leg. The automatic vaccination machines used in many parts of the world generally are designed for the neck injection. A skilled operator can vaccinate about 1600–2000 chicks/hour. A 20-gauge needle generally is used, as smaller gauge needles restrict the flow in cell-associated vaccines. Needles should be changed several times during the course of the day to prevent damage from burred or bent needles. Improper positioning of the chick or a bent needle can result in damage to the neck muscles or vertebrae. A dye is frequently mixed with the vaccine to allow visualization of the vaccine under the skin after injection. A quality check of technique generally means examining each bird in several boxes, 100 to a box, after vaccination looking for colored dye under the skin. The most frequent cause of missed birds is the operator trying to go too fast, resulting in chicks being pulled off the needle before proper deposition of vaccine.



Fig. 1.8. A modern hatchery with an egg injection system for in ovo vaccination.

Spray in the Hatchery

Spray vaccination of birds in the hatchery generally is done using a spray box that is triggered each time a box of chicks is placed inside or an in-line spray cabinet that sprays boxes as they move down a controlled speed conveyor line in an automated hatchery. Both methods, frequently used to deliver Newcastle disease virus, infectious bronchitis virus, or coccidiosis vaccine, attempt to mimic eye-drop vaccination. Spray vaccination in the hatchery generally works well if the droplets generated have a particle diameter of approximately 100–150 microns. Particle size is very important. Low relative humidity may decrease the particle size by the time it reaches the bird, resulting in too fine a spray. Fine spray, generally something less than 20 microns in diameter, can travel deep into the respiratory tract, resulting in excessive vaccine reaction if using a respiratory disease vaccine. Although there is some variability, Newcastle disease virus and infectious bronchitis virus vaccines are often delivered in 7 ml of distilled water per 100 chicks. Coccidiosis vaccines generally use more distilled water, approximately 20–25 ml per 100 chicks. Birds preening themselves and each other immediately following spray vaccination is thought to be important to the resulting vaccination response, although little data exists to support this concept.

Spray Vaccination on the Farm

With the increased acceptance and use of closed watering systems and the increased cost of labor required to effectively vaccinate through the drinking water, spray vaccination of respiratory vaccines, such as Newcastle disease virus and infectious bronchitis virus, has become increasingly popular. This method of vaccination frequently uses spray equipment adapted from insecticide and pesticide application technologies. As with the hatchery spray vaccination, the method is designed to mimic eye-drop vaccination but allows the vaccinator to avoid handling each bird in the poultry house.

Table 1.3. Visual analogy to droplet size diameter.

Analogy	Diameter (microns)
Wet fog	25–40
Visible droplets	50
Misty rain	50–100
Light rain	200–400

Distilled water generally is used to reconstitute the vaccine(s). Although the volume of water used varies depending on the spray machine selected, 5 gallons of water per 20,000 birds vaccinated is a good general recommendation. It generally is preferred to vaccinate a flock first thing in the morning. Fans should be turned off, if possible, and the lights should be as dim as the vaccinator can allow and still walk through the house. In floor houses, if another person is available, one person can split the flock while the vaccinator slowly sprays one side at a time. If possible, running fans should be minimized for the 15 minutes following vaccination.

An effective spray vaccination technique allows exposure of birds to aerosolized vaccine for approximately 5–10 seconds. This is best accomplished by spraying a relatively coarse spray, in the range of 100–150 microns, and walking slowly through the poultry house.

A visual evaluation of a spray pattern can be done with each vaccination. Look for an even distribution and consistent projection. A crude estimation of droplet size may be made using the analogies listed in Table 1.3 (62).

Intraocular or Nasal Drop in the Hatchery or on the Farm

Intraocular or nasal drop is a highly effective but labor-intensive method used to deliver respiratory disease vaccines for diseases such as laryngotracheitis. This method generally involves depositing approximately 0.03 ml of reconstituted vaccine in the eye or nares. Both techniques generally require the vaccinator to pause briefly as the vaccine disappears in the appropriate opening. A dye colored diluent helps to visualize the vaccine and allows a quality check on technique by looking around the nares or eye for dye. Frequently some dye can be seen by looking in the bird's mouth around the choanal cleft or edges of the tongue.

Drinking Water Vaccination on the Farm

A very common and useful technique in commercial poultry has been to apply vaccine through the drinking water. Proper preparation of the watering system to be used through removal of all disinfectants, such as chlorine, should be done two days prior to vaccination. It is best to buffer the system by flushing it with a weak solution of powdered skim milk, generally 1 cup powdered skim milk to 50 gallons of water (16). This type of buffer generally also is used while administering the vaccine.

Best results are achieved through a process that creates a mild degree of thirst by eliminating access to drinking water for approxi-

mately two hours prior to the vaccination procedure. This time varies widely. Climatic conditions may necessitate longer or shorter time periods. Thirst is optimal when the time between the first and last access to vaccine is approximately two hours. Two hours generally allows all birds, even those lower in the social order, adequate time to get a drink of water containing vaccine. This technique requires constant adjustment as the climate changes.

Wing Web Stab

Wing web vaccination requires individual bird handling but can be done relatively rapidly. There are two commonly used wing web application tools. The first is the traditional small plastic handle approximately 3 cm long that has two solid stainless steel prongs, approximately 2 cm long, with a bevel on each prong toward the needle end. The second newer application tool is referred to as a Grant inoculator. This tool has a self-contained reservoir for vaccine, most often fowl pox or fowl cholera, in which a needle passes through loading a new dose of vaccine for each bird inoculated. Both tools are designed to deliver approximately 0.01 ml on the needles to the bird's wing web. The wing web is an area that has relatively few feathers, bone, or muscle. The vaccinator loads the applicator and sticks the needle(s) completely through the skin on both sides of the web, originating from the underside of the wing. There is little or no bleeding, and vaccine has been inoculated through the needle holes. Wing web vaccination technique can be checked by returning to the vaccinated flock 7–10 days after vaccination and palpating the wing web area for nodular scabs or granulomas. These areas created by the vaccine are commonly referred to as "takes." Proper vaccination technique frequently results in 95–100% take.

Subcutaneous or Intramuscular Injection on the Farm

Subcutaneous and intramuscular injections are frequently used in breeder pullets and commercial egg-laying pullets prior to egg production. These vaccines are generally recommended for use at least 4 weeks prior to the onset of egg production to minimize any adverse effect the handling or the vaccine may have on egg production performance. Subcutaneous vaccination is most frequently performed using a 1/2 inch, 18-gauge needle, in the neck. The area half way between the head and the shoulder is optimal and allows the vaccinator to lift the skin away from the neck muscle and insert the needle, pointed towards the body of the bird, into the subcutaneous area and deposit the vaccine. Intramuscular injection generally is performed using a 1/2 inch, 18-gauge needle to inject vaccine into the breast or leg muscle. Breast muscle injections are safest when the vaccine is deposited in the superficial pectoral muscle 2–3 cm lateral to the keel bone. If the needle is kept at a 45-degree angle to the bird, any accidental injections into the body cavity or liver can be avoided (35). Leg vaccination generally is done in the lateral gastrocnemius muscle. Both intramuscular injection sites may result in residual emulsion being present for an extended period of time (21). A residual deposit in muscle depends on many factors including the antigen and the adjuvant found in the vaccine. Care should be taken to determine the intended use of meat before injecting intramuscularly.

Vaccine Failure

Numerous factors can cause a vaccine failure. One of the most common causes of vaccine failure is the inappropriate administration of the vaccine. Certain live vaccines, such as Marek's disease vaccine, are easily killed, and failure to follow the manufacturer's recommended handling practices will result in the inactivation of the virus prior to administration. Viable vaccines administered in the drinking water can, likewise, be destroyed before they reach the bird if they are mishandled or if water sanitizers have not been removed from the water prior to the addition of the vaccine. Vaccines that are administered by intramuscular or subcutaneous injection can also fail if vaccinators do not deliver the vaccine to the appropriate vaccination site.

Although the most common cause of vaccine failure is an inadequacy or error in vaccine delivery, numerous instances of vaccines simply not providing adequate protection have occurred. In some cases, the field strain of an organism is of very high virulence, and the vaccine strain is highly attenuated. In this situation, the flock may be effectively vaccinated, but the immunity is insufficient to protect against disease completely. Many infectious agents have several different serotypes, and vaccine failure may be the result of the antigens in the vaccine serotype being different and not providing protection against the particular serotype of the agent causing the field challenge. It is not uncommon for a vaccine break to occur with infectious bronchitis virus when the field challenge is of a serotype different from that of the vaccine used (8).

Management conditions play an important role in the prevention of vaccine failures. If infectious disease agents are allowed to build up on a farm over successive flocks without clean-out and disinfection, it is possible that the challenge dose of a particular infectious agent will be so great, or so soon, that a normally effective vaccination program will be overwhelmed. The immune status of the breeder flock also can be involved in a vaccine failure. If the breeder flock provides progeny with high levels of maternal antibodies, vaccination during the first 2 weeks of life may result in the vaccine being neutralized. The timing of the vaccination of young poultry with viable vaccines must always take the presence or absence of maternal antibodies into consideration.

Certain infectious disease agents and mycotoxins are immunosuppressive and may result in vaccine failure. Infectious bursal disease virus (Chapter 7), infectious anemia (Chapter 8), and Marek's disease virus (Chapter 15) are examples of agents that may cause severe immunosuppression in chickens. One mycotoxin, aflatoxin, has been shown experimentally to be immunosuppressive and has been implicated in decreased resistance to disease (see Chapter 32).

Monitoring a Vaccination Program

Methods for evaluating the effectiveness of a vaccination program varies widely and generally involves evaluating and monitoring overall health. Frequently, absence of morbidity and mortality is used as a criteria for success. In areas of endemic challenge with very pathogenic organisms, such as viscerotropic velogenic Newcastle disease virus, an ineffective program is very

obvious due to clinically ill or dying birds. However, in most areas of the world, a suboptimal program is much less obvious. In this case, an effective program must minimize the risk associated with disease and maximize production efficiency as economically and practically as possible. Many aggressive vaccination programs aimed at high levels of protection are detrimental to growth efficiency and expensive. The goal of veterinarians and other health professionals is to balance these criteria as efficiently as possible.

Performance Parameters

Metrics generally used to judge overall health, which encompasses vaccine program efficacy, are culls at the hatchery, 7-day mortality, 14-day mortality, final flock livability, feed conversion efficiency, rate of gain, condemnation, egg production, and egg quality. Many of these metrics have standards or comparative histories established through each company's own historical data or, in the United States at least, national reporting services such a AgriStats (AgriStats, Fort Wayne, IN), or Agrimetrics (Agrimetrics Associates, Inc., Midlothian, VA), and government reporting services such as the poultry slaughter reports published monthly by the National Agricultural Statistics Service (NASS), Agricultural Statistics Board, U.S. Department of Agriculture. An additional metric that can be used over time is antimicrobial and antiparasitic drug usage. Although this is influenced by many things, including management changes and climatic shifts, monitoring usage is an essential piece of evaluating overall health and vaccination program efficacy.

Examination of Field Birds

Health surveys (6, 33) that include extensive gross and microscopic evaluation of necropsy specimens and controlled challenge studies (46) to measure a relative protection level are both useful in assessing vaccine program effectiveness. Perhaps the most frequent or routine controlled challenge work is done to measure passive protection of broiler chicks from hens hyperimmunized to infectious bursal disease (46). Trends in program efficiency may be identified over time if sufficient groups of chicks are sampled.

Serologic Monitoring

Serologic monitoring (60) is only useful in production medicine if adequate samples have been analyzed over time in order to establish a normal baseline for a specific program, in a specific location, in a specific bird, using specific and consistent application techniques, with samples run consistently by a specific laboratory. After a baseline is established, flocks can be identified, that have serologic profiles above or below the established baseline.

In broiler and turkey production flocks, an effective monitoring program can be the regular sampling and testing of blood as they are slaughtered at the processing plant. This serologic monitoring will establish a baseline of antibody titers that are the result of both vaccination and field challenge. Changes in the usually observed antibody titers may indicate a decrease in the efficacy of vaccine administration or an increased field challenge by a particular pathogen. A regular serologic monitoring program is also helpful to determine whether a flock has been exposed to a new pathogen, not previously present in the region.

Serologic monitoring of layer flocks should be performed before the flock is placed in the layer building, with periodic serologic monitoring throughout the production cycle. This type of program will assess both the efficacy of vaccine administration and the disease challenge the flock experiences in the field. Breeder flocks should be monitored in the same way as layer flocks and, in certain instances, breeders can be revaccinated during production to boost the maternal antibody titers of their progeny if they are found to be low.

Interpretation of Serologic Data

It is usually impossible to differentiate between antibodies that are produced by vaccination versus those induced by field exposure to a given infectious agent. The only difference that may be observed is that the antibody titer following a field challenge may be higher than that observed following vaccination. A valid interpretation of serologic results requires a complete knowledge of the flock's vaccination history.

It usually takes poultry 1–3 weeks to produce detectable levels of antibodies in their serum. It is possible, therefore, to collect blood during the middle of a disease outbreak and not be able to detect any antibodies to the causative disease agent. If this same flock is tested 2 weeks later, however, serum-antibody levels will be high. A useful practice in establishing a disease diagnosis is to take acute and convalescent serum samples from the flock as it is undergoing an unknown disease challenge. Typically, the acute serum sample collected during the initial phase of the disease outbreak will be negative for antibodies to the suspected disease agent. The convalescent serum sample, taken shortly after the flock has recovered, if positive, will provide a definitive diagnosis when interpreted in conjunction with the clinical signs and lesions of the case. An important concept in the interpretation of serologic results is that a single positive serologic test only indicates that the flock was exposed to that disease agent during its life.

Different laboratories often conduct serologic tests using different reagents or techniques. Because of this, comparing antibody titers (a titer is a measure of the level or concentration of antibody in the serum) reported from different laboratories may be confusing. It is best to use one laboratory for a given test so that a familiar range for negative, low, or high titers is established. With experience and training, production managers can become skilled at the interpretation of serologic results.

Flock Profiling

Today's disease problems often represent the sum of various subclinical disorders occurring at different times throughout the life of a flock. Acquisition of the fullest understanding of this sequential collection of serologic and other data concerning multiple pathogens requires disciplined and careful organization. The systematic, graphic presentation of this data is commonly called a "flock profile." The establishment of such profiles is facilitated by enzyme-linked immunosorbent assay (ELISA) technology,

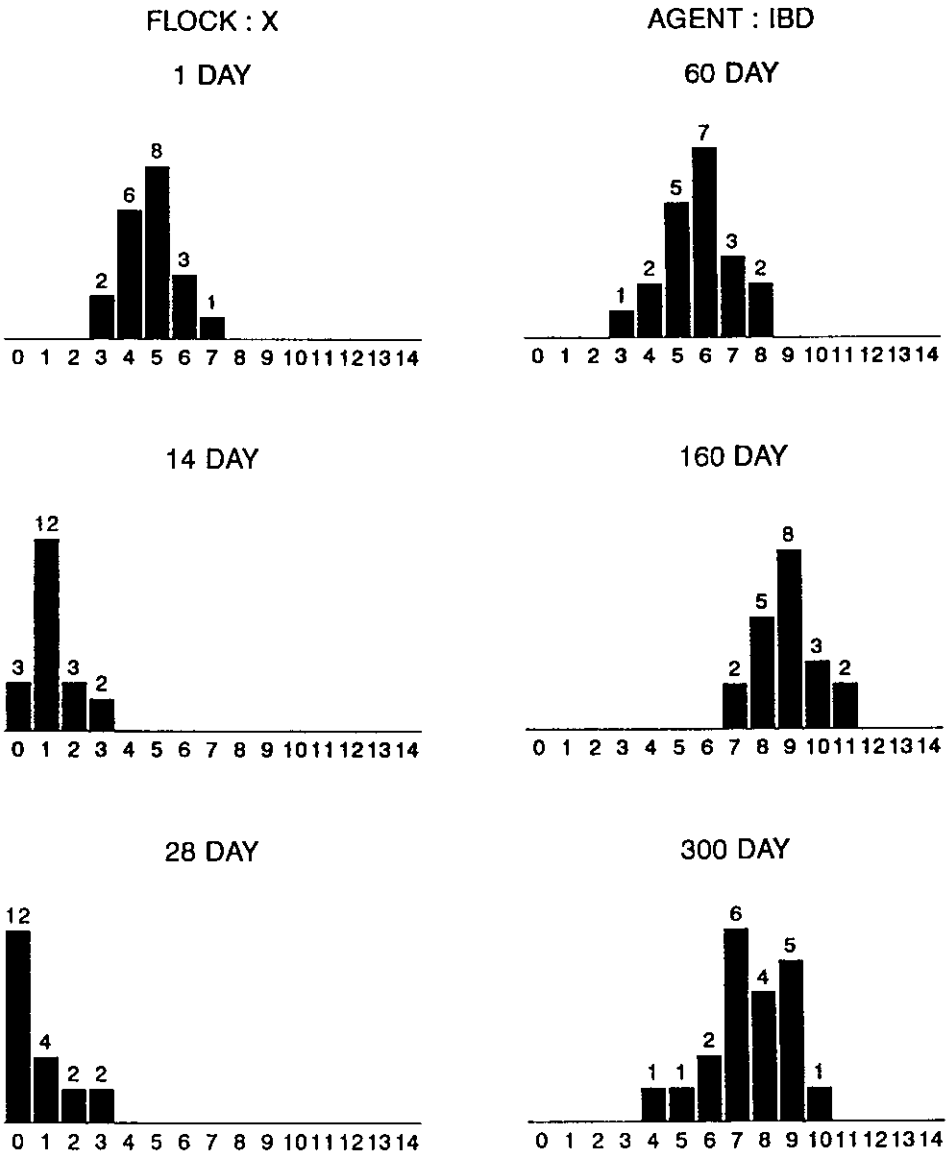


Fig. 1.9. Temporal graphic distribution of infectious bursal disease (IBD) enzyme-linked immunosorbent assay (ELISA) group titer levels at 1, 14, 28, 60, 160, and 300 days of age for an IBD-vaccinated broiler breeder flock. Numbers on the X-axis represent group titer levels obtained by ELISA. Titers of 0 are group 0; 1–350 are group 1; 351–1500 are group 2; 1501–2500 are group 3; 2501–3550 are group 4; etc., with titers of 12,500 comprising group 14. Numbers above each bar represent the number of samples reacting at each level on the indicated day of age.

because a single basic test system is used to monitor for a broad array of diseases (59).

Snyder *et al.* (61) demonstrated the value of correlating ELISA profiling data with flock performance. The further evolution and diagnostic advantages of the graphic presentation of ELISA-based flock profiling data in combination with gross and microscopic pathology data was described by Mallinson *et al.* (39). The method has broad applicability to epizootiologic investigations, field research, and quality control. Baseline profiles can be established both as targets for vaccination goals and as a base from which deviations from the norm may be demonstrated when a field problem is subsequently encountered. Several flock-profiling kits and systems are now commercially available. Their value is enhanced when good data retrieval and graphic presentation of data (Fig. 1.9) is combined with the diagnostician's veterinary skills and experience in assimilating medical information and establishing a plausible diagnosis.

Sanitary Environments

Grounds around Buildings

Rodent Control

Piles of trash and unused equipment are good hiding and breeding places for rats, mice, and ground squirrels, which may serve as reservoirs of disease and contaminate troughs with their excrement. Rodents are reluctant to travel over open spaces that do not provide protective cover. A 20-m band of short-mowed grass or gravel tends to discourage the migration of rodents into a poultry building from surrounding areas. Feed spilled or left in stored troughs is an attractive food supply; when it is exhausted, rodents will find any avail-

able route into the building where they have intimate contact with poultry. Even if buildings are rodent-proof, excrement can be tracked in on footwear. It is more difficult to get rid of rodents once the premises are infested than to keep them out initially.

Insect Control

Many parasites and disease agents are harbored from one generation to another in resident insects (Marek's disease), require an insect for an intermediate stage of development (tapeworms), or are simply carried from bird to bird mechanically or by biting (fowl pox virus). Countermeasures against insects are part of the sanitary environment and cleanup.

Some methods used to keep insects away from buildings are an apron of treated soil to prevent growth of all vegetation, an apron of hard surface material, or a border of well-mowed green grass. Spraying the area around buildings with an insecticide also prevents insect buildup, but the other methods have the additional advantage of reducing fire hazards to the buildings.

A good practice during cleanup is to spray the grounds, litter, and buildings with an insecticide immediately after removing fowl and then allow a few days for effective insect kill before removing litter preparatory to cleaning and disinfection. This is especially important when there is a history of an insect-borne disease in the previous brood. After cleaning, the building should be sprayed again with an insecticide having a residual effect to prevent reinfestation. Professional, integrated rodent and insect control services are available in some locations. They may provide cost-effective convenience.

Dead-Bird Disposal

Foci of Infection

When birds die owing to disease agents, carcasses remain a source of infection for penmates and other poultry on the same or other farms. Also, hopelessly sick birds discharge infectious material into the environment and should be removed from the flock and killed in a manner that will not permit the discharge of blood or exudates (see "Diagnostic Procedures"). Whether the result of a serious clinical infection or just the usual expected mortality, all carcasses should be disposed of by one of the following methods to prevent dissemination of disease. Whatever method is used must be a practice that is allowed by state and federal environmental authorities.

Rendering

Freshly dead poultry, like livestock, can be rendered into fertilizer or other products. The rendering temperature should be sufficient for sterilization, and the truck bed used to transport the carcasses washed and disinfected. Cans used to haul the carcasses should be steam-cleaned and sterilized. Again, it should be remembered that commercial or contract haulers of dead carcasses may introduce another disease from some other outbreak unless strict precautionary measures are taken.

Incineration

Incineration is the most dependable way of destroying infectious material. Many smokeless, odorless incinerators for disposal of animal carcasses are available commercially. These devices are expensive to purchase and operate but are suitable in some instances.

Burying

For losses creating a serious disposal problem, where environmental regulations allow, a deep hole may be dug and the carcasses buried so animals cannot get at them. The best and easiest way is to use a backhoe and dig a deep narrow trench. Each day's collection of dead birds can be deposited and covered until the trench is filled.

Pit or Tank Disposal

For small losses and normal attrition, a decomposition pit can be used (Fig. 1.10A). A bigger and less elaborate one than that shown can be built, but precautions should be taken to ensure that it is not located where it will contaminate drinking water supplies, that the roof or walls will not cave in, that animals will not dig into it, that flies and other insects cannot get into it, and above all that children cannot fall into it. The pit cover should be sealed with tar paper or plastic and be strong enough to hold at least a foot of soil overlay. Where ground water levels are close to the surface (deltas, lowlands, and shorelines), underground pits may be undesirable.

Composting

Aerobic, thermophilic batch composting of poultry carcasses is a method of disposal developed at the University of Maryland (43). Compost mixtures of straw, whole poultry carcasses, manure, and water in the proportions of 1:1:1.5:0.5, respectively (1/3 of water added to each layer), decompose rapidly and odorlessly. Composts heat rapidly, attain temperatures of between 145–165° F, and reduce soft tissues completely within 14 days. Compost structures and management procedures are simple (Fig. 1.10B). Pathogen survival studies suggest the process is biologically "clean." Attempts to isolate coliform and *Salmonella*-like bacteria and IBD virus have yielded negative results. Composting may be an effective alternative to more traditional dead-bird disposal methods, especially where water tables are near the surface.

Buildings and Runs

Clean Buildings

A clean sanitized environment is good insurance against disease outbreaks from any cause. Stringent sanitary practices are frequently ineffective because disease is tracked in after the buildings and equipment are cleaned and disinfected, or because some step in the total program was omitted, and a focus of infection was preserved.

Litter Removal

When a house is depopulated, the litter or droppings should be removed preparatory to cleaning. With development of huge specialized poultry farms, proper and economical disposal of litter and poultry manure has become a serious problem. There is no clear-cut answer. A general recommendation is to remove it far enough from the buildings so that insects will not crawl or fly

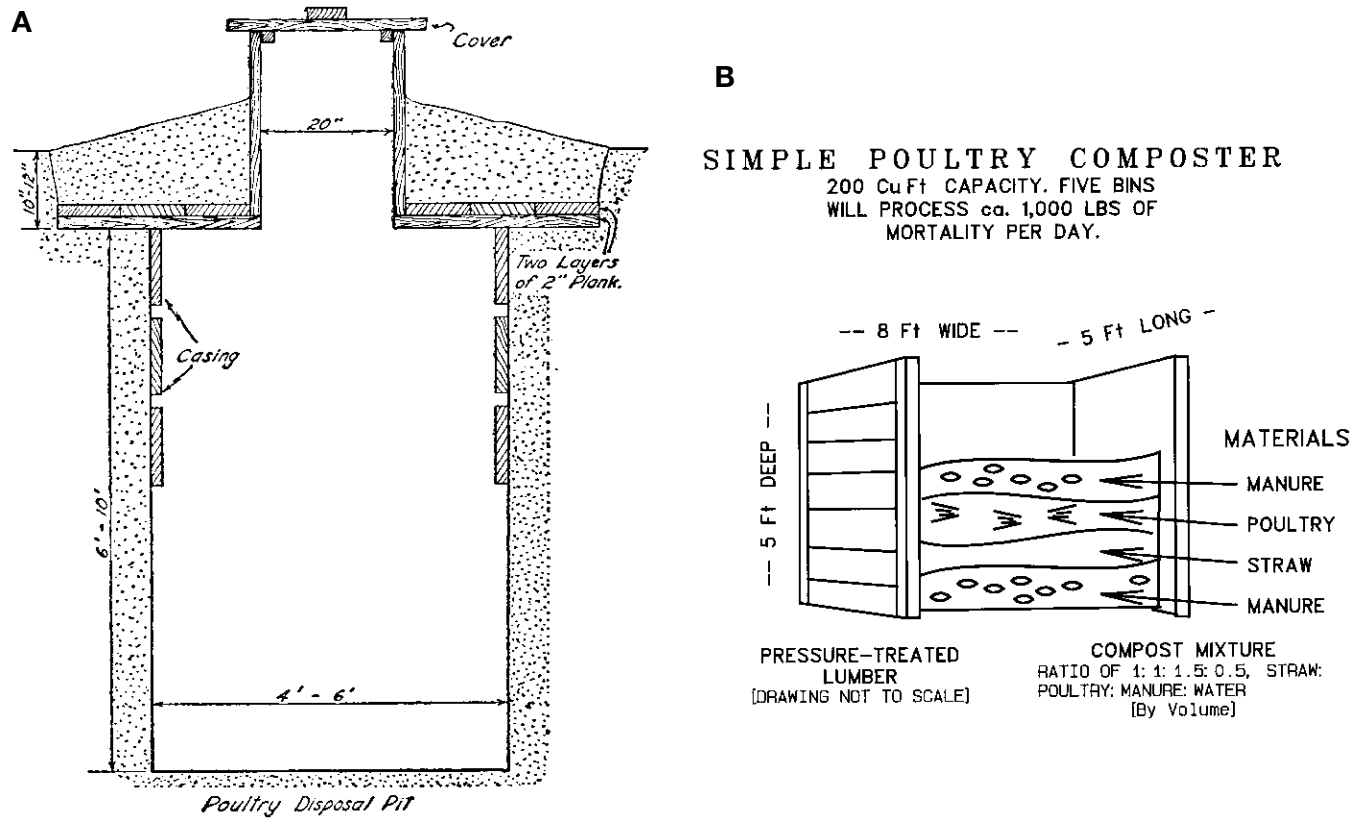


Fig. 1.10. A. Poultry disposal pit. Such a pit can be made any size that is convenient. B. A simple above-ground poultry carcass composter of 200 ft^3 (5.7 m^3) capacity. Five such bins will process 1000 lb (455 kg) of carcasses per day. (Courtesy Poultry Science Dept., University of Maryland)

back into the houses, and to dry it, compost it, or spread it onto fields and work it into the soil. If cleaning is done while chickens are still present (cages), remember that contracted personnel, trucks, and equipment may recently have been on another farm where a disease outbreak occurred.

In some cases, the nature of a disease may dictate that some extra precautions (wetting down or soaking with disinfectant, delaying removal, burying, burning) be taken with litter, even though expensive. Any treatment of manure or litter must consider residual effects of the applied compounds on plant life when treated manure is spread on the land. For most disease agents, composting of litter or droppings is sufficient. Whatever is done, one must be aware that wherever litter is spilled or piled, it remains as a disease reservoir for varying lengths of time.

Outside Runs

In the case of outside runs such as turkey and game bird ranges, the topsoil should be scraped off and hauled some distance from the poultry. Sunlight and soil activity combine over a long period to destroy most pathogens. Anything that can be done to aid the destruction process is helpful. Removal of organic residues, such as leaf beds and manure accumulations, helps to reduce the danger for future broods. It is best to rotate the ranges or dirt yards so that they stand idle for one complete flock cycle.

Washing and Disinfecting

After the litter or cage droppings have been removed, cleaning and transfer equipment, feeders, waterers, egg collecting equipment, walls, floors, roosts or cages, outside concrete or suspended runs, and entries to buildings should be washed thoroughly and disinfected. If the supply of water is limited and washing is not possible, dry cleaning may suffice if it is thorough and includes scraping and sweeping or vacuuming surfaces, corners, ledges, nests, and feeders. The amount of disinfectant used on dry-cleaned surfaces must be increased over that required for washed surfaces.

If possible to do so efficiently, it is preferable to clean the house without removing equipment. If not, all portable equipment should be removed, soaked with water, then thoroughly washed and dried. A high-pressure water hose is effective. Equipment that cannot be removed should be washed in place and then the entire inside building surface washed clean. If the building has been constructed to facilitate good cleaning, it can be done easily. If not, satisfactory cleaning may not be accomplished at all or only with great effort and expense. A large concrete apron equipped with racks and a high-pressure hose is a good place to clean and stack equipment.

After washing, disinfection is in order (see "Disinfectants"). Many effective disinfectants are sold under a variety of trade names; follow the manufacturers' recommendations. The impor-

tant thing is that the surfaces be clean before application. Disinfectants applied to dirt-encrusted surfaces are ineffective and wasted. Not only are they inactivated by organic material in the dirt, but they never reach the infectious agents beneath it. Thorough washing removes most infectious agents from the house and equipment and leaves a clean surface so the disinfectant can reach those that remain. Two to four weeks of idleness or "down time" before a new flock is moved in is additional insurance against carryover of disease; however, down time should be considered as an adjunct to, and not a substitute for, thorough cleaning, washing, and disinfection.

Built-up Litter and Uncleaned Buildings

Commercial producers demand chicks and poults that are free of pathogenic microbial agents acquired through egg transmission or from unsanitary hatchery or delivery environments. To maintain this status, it is preferable to place these healthy new flocks in cleaned and disinfected buildings with fresh clean litter. Providing these ideal conditions is expensive because of labor and litter costs. Also, suitable litter materials are becoming less plentiful. In keeping with the constant necessity to reduce production costs and cope with shortages, rearing of several successive flocks on the same (built-up) litter has become an economically acceptable practice with broilers, where the life span is very short and single ages of birds per farm permit complete depopulation at the end of each brood. The litter in turkey grow-out buildings is also frequently used for several successive flocks. This trend has become commonplace with the use of litter-processing machinery, which can break up caked litter and produce a litter with acceptable production characteristics. The continual reuse of such built-up litter will result in an increase of microbial pathogens and parasites within the litter. Commercial producers recognize, however, that cleaning and disinfecting a house or group of houses may become necessary any time excessive economic losses are attributable to a disease that may carry over to the next brood.

The practice of reusing litter is much less attractive for rearing egg-production flocks, where the life span usually exceeds 18 months; it is not acceptable for rearing breeding flocks that produce hatching eggs for new generations. In any case, those who reuse litter should be fully aware of the possible hazards involved and should follow other sound disease control practices to minimize the dangers.

When old litter must be reused, it is good insurance to remove any caked or excessively fouled litter, accumulated feathers, and decomposed carcasses. A layer of fresh clean litter then should be placed under the heating brooders and over the area to which the young will be confined or will spend most of their time the first weeks of life. One disadvantage of multiple brooding on the same litter is the excessive dust that accumulates. Inhalation of the dust provides an avenue of entry to the respiratory tract for bacteria and fungal spores.

Disinfectants

To disinfect is to free from pathogenic substances or organisms or to render them inert; a disinfectant is an agent or substance that disinfects chiefly by destroying infective agents (pathogenic microorganisms) or rendering them inactive; disinfection is the act or process of destroying pathogenic microorganisms. To sanitize is to reduce microbial populations and keep them from multiplying.

Properties

Among the properties of an ideal disinfectant are low cost per unit of disinfecting value, ready solubility in hard water, relative safety for humans and animals, ready availability, nondestructibility to utensils and fabrics, stability when exposed to air, absence of objectionable or lingering odor, no residual toxicity, effectiveness for a large variety of infectious agents, and no deleterious accumulation of any portion of the disinfectant in meat or eggs. For any disinfectant to be effective in economical quantities it must be applied to surfaces that have first been freed of debris and organic material by thorough scraping, scrubbing, brushing, and dusting and washing with soap or detergent solutions. Many disinfectants are highly efficient but only when these basic cleaning prerequisites have first been met.

Types

Many disinfectants of similar composition are sold under different trade names. Before buying a product with an unfamiliar name, compare types and values with a well-known product. Directions for dilutions given by the manufacturer should be closely followed. Complete discussions of various disinfectants and sterilization methods should be consulted (12, 52). Additional references on disinfectants and their use (28, 48) and textbooks on pharmacology and therapeutics should be consulted.

The virucidal activities of several commercial disinfectants against velogenic viscerotropic Newcastle disease have been determined (73). A list of 99 commercial disinfectants approved for use against avian influenza virus is available from the Environmental Protection Agency (USA). The list provides product names, basic formulations, and dilutions along with the names, addresses, and telephone numbers of appropriate distributors or formulators.

Phenol (Carbolic Acid)

Phenol is a chemical substance obtained from coal tar. In its pure form, it occurs as colorless crystals having a characteristic and familiar odor (lysol soap). It usually is sold in water solutions and is too expensive for general poultry house use. It is, however, the chemical used as a basis for determining the phenol coefficients of various disinfectants (relative ability to kill specific test organisms when compared with that of phenol). O'Connor and Rubino (45) present a complete discussion of the phenolic compounds. Commercial disinfectants containing phenolic compounds have been developed and marketed at cost values, which permits wider use in poultry operations. Some have residual activity persisting after they have dried, giving the advantage of continued suppression of bacterial and viral populations on sprayed surfaces.

Cresols

Cresol extracts of coal tar products are compounds closely related chemically to phenol and having similar bactericidal prop-

erties. They are thick yellow or brown liquids, miscible with water but only slightly soluble. They form the basis for a large number of commercial brands made by combining cresol with soap.

Bisphenols

Bisphenols are compounds composed of two phenol molecules modified and joined by various chemical linkages. Halogens, particularly chlorine, have been combined with bisphenols to increase their effectiveness; some of the chlorophenols have high antifungal activity. Bisphenols are frequently combined with other phenolic compounds in disinfectants. Additional information on these compounds is available (45).

Pine Oil

Pine oil has proved satisfactory as a disinfectant and has the advantage of being less injurious to the skin than cresol compounds. The odor is also less objectionable and in fact is rather pleasant, which enhances its desirability for use in offices and lavatory areas. Because it is insoluble in water, it is used in the emulsion form with soap or other emulsifying agent.

Hypochlorites and Chlorinated Lime

Chlorine is the basis of disinfectants known as hypochlorites, which contain about 70% available chlorine. Hypochlorites (22) are available as powders containing calcium hypochlorite and sodium hypochlorite (NaOCl) combined with hydrated trisodium phosphate and as liquids containing NaOCl. Chlorinated lime (bleaching powder), prepared by saturating slaked lime with chlorine gas, was one of the earliest recognized disinfectants. It has been largely supplanted by the more readily available hypochlorites.

Products containing NaOCl are essentially liquids ranging in concentrations from 1–15%. The 15% solutions are used to prepare 5% solutions with water for bleaches and sanitizing agents. Germicidal potency of hypochlorites is dependent upon concentration of available chlorine and the pH (acidity) of the solution or upon the amount of hypochlorous acid formed, which, in turn, is dependent upon both factors. The influence of pH, especially in dilute solutions, is even greater than the percentage of available chlorine. Increasing the pH decreases the biocidal activity of chlorine, and decreasing the pH increases the activity. Germicidal activity is speeded up by raising the temperature.

If used according to directions, hypochlorites are highly efficient. Their principal use in the poultry industry is for egg washing and sanitizing and for disinfecting limited areas such as incubators, incubator and hatcher trays, and other areas around the hatchery, egg breaking areas, small brooders, and water and feed containers. They can also be used on cement surfaces. All surfaces to be disinfected with hypochlorite solutions must first be thoroughly cleaned to ensure the greatest efficiency. Stock supplies should be kept in dark, cool places, and containers should be tightly sealed when not in use. Fresh solutions must be prepared daily and periodically tested to ensure that proper levels of available chlorine remain. A simple swimming pool test kit may prove helpful for such monitoring. Recently purchased or stored hypochlorites have been found to have wide ranges of concentration values.

All products containing chlorine must be handled with care because free chlorine is destructive to fabrics, leather, and metal.

Organic Iodine Combinations

Iodine has long been recognized as an effective disinfectant. Many of the disadvantages of earlier products have been overcome by combining iodine in organic complexes, sometimes called tamed iodine. The term *iodophor* refers to a combination of iodine with a solubilizing agent that slowly liberates free iodine when diluted with water. The term most frequently refers to formulas consisting of iodine complexed with certain types of surfactants that have detergent properties. These complexes are said to enhance the bactericidal activity of iodine and render it nontoxic, nonirritating, and nonstaining when used as directed. The detergent also makes the products water soluble and stable under usual conditions of storage. No offensive odor exists, and the detergent properties impart cleansing activity. See Gottardi (30) for additional information on iodine compounds.

A group of commercial iodophors have been developed and marketed for a wide variety of disinfectant uses. Some of these products have a built-in indicator of germicidal activity; as the solution is used up, the normal amber color fades. When the solution is colorless, it is no longer effective. The products can be mixed in cold and hard water. Organic iodine products have a wide variety of uses in the industry. They can be applied without hazard to nearly all surfaces and are useful for disinfecting hatchery and incubator surfaces, incubator and hatcher trays, egg breaking areas, feeders and fountains, footwear, and poultry buildings. Like other disinfectants, these compounds are most effective on clean surfaces.

Quicklime (Unslaked Lime, Calcium Oxide)

The action of quicklime depends on liberation of heat and oxygen when the chemical comes in contact with water. On the poultry farm, its use is limited to small yard areas that are damp and cannot be exposed to the sun, disinfection of drains and fecal matter, and whitewashes. As quicklime has a caustic action, birds should be kept away from it until it has become thoroughly dry.

Formaldehyde

Formaldehyde (CH_2O) is a gas. It is sold commercially in a 40% solution (37% by weight) with water, under the name of formalin. It may also be purchased in the form of a powder known as paraformaldehyde (paraform, triformal, formaldegen). When heated, this powder liberates CH_2O. A suitable heating device is a thermostatically controlled electric pan with a timer that can be controlled from outside the fumigation chamber. Manufacturer's directions on amounts to use for each type of equipment and the means of liberating the gas must be carefully observed.

Formaldehyde is often generated by adding formalin to potassium permanganate ($KMnO_4$) in an earthenware crock or metal container. Because of the heat generated by the chemical reaction, glass containers should not be used. The container should be deep and have a volume several times that of the combined chem-

icals, because considerable bubbling and splattering takes place. The ratio in liquid measure of formalin is approximately twice the dry measure of $KMnO_4$ (1 g $KMnO_4$/2 mL formalin). If too much formalin is used, the excess will remain in the vessel. If too much $KMnO_4$ is used, the excess remains unchanged and is wasted. Potassium permanganate is poisonous. Both these compounds must be kept in accident-proof containers in a safe place away from work traffic.

A suitable fumigation cabinet must have a source of heat, a fan to circulate the warm humid air and fumigant, a source of humidifying moisture, and a method of generating formaldehyde gas. The box should be airtight and have an exhausting device from the fumigation box to the outside of the building. It is much safer to locate fumigation chambers outside of any building and away from human traffic.

Although it is a powerful disinfectant, CH_2O has many disadvantages, especially its volatility, pungent odor, caustic action, and tendency to harden human skin—properties that make it disagreeable to apply. It is extremely irritating to the conjunctiva and mucous membranes, and some people are very sensitive to it. Because of this and other toxic properties, precautions must be taken to prevent its escape into areas where people work. Its chief advantages are that it can be used as a gas or vapor for fumigation of hatching eggs. It is a good disinfectant in the presence of some organic matter, and it does not injure equipment with which it comes in contact. The maximum atmospheric concentration permitted in work areas by some Occupational Safety and Health Administration (OSHA) regulations is 2 ppm with a maximum 15 minute exposure time. Suitable gas masks should be readily available near fumigation boxes. Formaldehyde can be neutralized with ammonium hydroxide by using a solution of approximately 30% and a quantity not to exceed one-half of the quantity of formalin used in the fumigation. Ammonia may be released by sprinkling or spraying in the intake air during evacuation of the fumigation box after the surfaces have dried completely.

Formaldehyde gas is widely used in poultry enterprises for fumigation of hatching eggs to destroy potential pathogenic shell contaminants. It is also used at the end of cleanup to fumigate the inside of incubators and hatchers and their contents.

Fumigation of incubators and eggs has been an established practice in the industry and has varied little over the years. Various recommendations have been made for quantities, humidity, temperature, and time for adequate sterilization of shells of hatching eggs. Frequent recommendations specify the following: 60 g $KMnO_4$:120 mL formalin/100 ft^3 (2.8 m^3) cabinet space, 21.1°C, 70% humidity, and 20 minutes fumigation time. The higher the humidity and temperature, the more effective the fumigation. When fumigation is completed, exhaust ducts are opened and the gas thoroughly exhausted before anyone opens the door to the cabinet.

In modern enterprises, hatching eggs frequently are handled only once and are placed directly into plastic holders (flats), which then travel in stacks through the fumigation, transportation, and storage route and eventually into the incubators. Entire racks, dollies, or pallets of closely stacked flats of eggs are thus fumigated in large boxes. To generate adequate concentration of CH_2O and have it penetrate and disinfect the egg shells in the centers of these stacks, there should be an increased quantity of chemicals (75 g $KMnO_4$:150 mL formalin/100 ft^3), higher humidity (up to 90%), higher temperature (up to 32.2°C), longer time (up to 30 minutes), and vigorous agitation of the gas during fumigation so that it will penetrate the spaces and effectively sanitize surfaces of eggs in the centers of such large stacks. Pressed paper egg flats tend to trap CH_2O and continue to release the gas during storage and processing; therefore, CH_2O fumigation should be confined to eggs in plastic flats or wire containers.

CH_2O fumigation is sometimes used to disinfect the inside and contents (including eggs at 18 days of incubation) of hatching machines. Because these machines are inside of the building, this should not be done unless provision is made for adequate ventilation of the gas to the outside of the building when fumigation is completed.

Certain precautions are necessary after fumigation of hatching eggs. The incoming air for exhaustion must be clean, otherwise the humid surface of the egg can become recontaminated. During extremely cold weather, outside air must be warmed before entering the fumigation chamber to avoid overchilling eggs. Although humidity is essential for disinfective activity of the CH_2O, the surface of eggs should not become visibly wet during fumigation and should be dry when the eggs leave the fumigator.

Fumigation should not be done in incubators because of the danger of injuring embryos. Also, it should not be done at such a high concentration after the hatch begins because of the danger of injuring chicks or poults. Formaldehyde may be generated in hatchers by using approximately 20 mL formalin solution/100 ft^3. The formalin is soaked into enough cheesecloth so that it does not drip, and the cloth is hung in the circulating currents in the box. Effectiveness of this method is limited because of the low concentration.

Antifungal Imidazoles

The use of formaldehyde may pose some health and safety concerns for hatchery personnel. An effective substitute for formaldehyde used to control *Aspergillus* spp. in the hatchery is imazalil or enilconazole (nonproprietary names for phytopharmaceutical and veterinary uses, respectively) (70). The imidazoles are fungistatic at low concentrations by inhibiting ergosterol synthesis and fungicidal at high concentrations by causing direct membrane damage (55). Imazalil is intended for use on clean hatchery surfaces or equipment and is delivered in an aqueous spray or by smoke propellant canisters. The antifungal properties of imazalil must be complemented with the use of antibacterial disinfectants for a complete hatchery sanitation program.

Copper Sulfate (Bluestone)

Although copper sulfate ($CuSO_4$) and other salts of copper have a marked toxic effect upon some of the lower forms of life, they are not considered good general disinfectants. Copper sulfate is toxic to algae and fungi and has been used in attempts to stop or prevent outbreaks of fungal diseases. It has been used in the feed at 0.5 lb/ton and sometimes 1 lb/ton for short periods without noticeable toxicity to chickens. Poultry usually will drink water containing $CuSO_4$ at no greater concentration than 1:2000, but a

concentration greater than 1:500 may be toxic when given in the only source of water. Turkeys do not like water containing $CuSO_4$ and will seek other supplies if available. A 0.5% solution may be of value for disinfecting feed hoppers, water fountains, and surrounding areas associated with outbreaks of fungal disease.

Quaternary Ammonium Surfactant Disinfectants

Quaternary ammonium products (quats) are considered to be good disinfectants when used according to directions. They are noncorrosive, water clear, odorless, cationic (+ charged ions), nonirritating to the skin, good deodorants, and have a marked detergent action. They contain no phenols, halogens, or heavy metals and are highly stable and relatively nontoxic. Most quats cannot be used in soapy solutions. All surfaces to be disinfected must be thoroughly rinsed with water to remove any residue of soap or anionic (– charged ions) detergent before using quats for sterilizing purposes. Some hard-water minerals may interfere with their action. See Merianos (42) for more information on these compounds.

Quats are used for washing eggs and disinfecting hatchery surfaces, incubator and hatcher trays, egg breaking equipment and areas, feeders and waterers, and footwear, among other uses.

Sunlight and Ultraviolet Radiation

Solar radiation has disinfecting properties; however, because the material to be treated must be in thin layers and exposed to direct rays, this method is limited to impervious surfaced yards, concrete and blacktop aprons, and equipment that can be thoroughly cleaned before being exposed. The construction of most poultry houses prevents efficient disinfection by the sun. A cement platform fully exposed to the sun makes a convenient place for treating movable equipment. If properly constructed with a drain, such a platform can be used as a washing and disinfection rack. A concrete apron before the poultry house entry will be washed by rains or can be washed by hose to take advantage of the disinfecting power of the sun's rays on the clean surface.

There are many types of germicidal (ultraviolet or UV) lamps, but not enough scientific evidence is available to warrant a recommendation for their general use in hatcheries or on poultry farms. A complete review of the use of UV radiation in microbiologic laboratories is available (47).

Hot Water

Hot water adds to the efficiency of most disinfectants, and if applied in the form of boiling water or live steam, is effective without the addition of any chemical. Detergents added to systems for generating and disseminating hot water and steam will increase cleaning and decontaminating efficiency. Live steam must be applied directly and at close range to the part to be disinfected.

Dry Heat

Dry heat in the form of a flame is effective if the flame comes in contact with the pathogen to be killed. All methods involving direct flame are fire hazards and not recommended except possibly on cement surfaces. In tightly controlled circumstances, flames might be used to eliminate hard-to-remove feathers and fluff accumulations.

Other commercial disinfectants, mostly organic compounds, are available under trade names. Many are combinations of several individual disinfectants with complementary properties. Some also have long residual activity. To choose disinfectants wisely, one must continually keep abreast of new product development through current scientific and lay publications.

Residues of disinfectants used to sanitize drinking fountains should be rinsed off with fresh water before water vaccines are given, because they can inactivate the vaccine virus.

Pesticides (Insecticides and Parasiticides)

Properties

The Environmental Protection Agency (EPA) defines a pesticide as any substance intended for the preventing, destroying, repelling, or mitigating of any pest. A pest can be any insect, animal, plant, or microorganism. Insecticides destroy animal parasites such as lice, mites, ticks, and fleas. They also destroy other undesirable insects (flies, beetles, ants, and sow bugs) in the environment. Some insecticides are highly toxic to humans and livestock. Their use (preferably by a licensed expert as part of a professional, integrated insect and rodent control service) is recommended only as an adjunct to a properly conducted total sanitary control program. Many disinfectants are also destructive to lice, mites, and other similar parasites but must come in contact with them. Most pesticides, however, are useless as disinfectants.

Suitable insecticides are those that can be used on or around poultry without causing toxic effects to humans or birds from contact or ingestion and that do not accumulate to harmful levels in edible tissues or eggs as a result of ingestion or absorption.

The list of available and permissible commercial insecticides has declined greatly and changes frequently. Two extension bulletins, *Livestock and Livestock Building Pest Management* and *Poultry Pest Management,* are available from the Ohio State University Extension. Many, widely used in the past, have been prohibited for use around food animals because of the deposition of insecticides in fatty tissues and eggs. Others have been abandoned because populations of insects became resistant to them. It is necessary, therefore, to keep informed on available effective insecticides through current government, university, and industry literature. In some situations, it may be cost effective to contract this complex changing activity through an agency providing professional insect and pest control services. Biosecurity measures for the agency employees and equipment must be considered when contemplating such contract services.

The limited number of available commercial parasiticides, their active chemical properties, limitations, tolerances, and various applications, are discussed in detail in Chapter 26. See also Chapter 32 for toxic effects of some insecticides.

Unlike flies, which travel to insecticide baits or over insecticide-treated surfaces, bird ectoparasites are best controlled by bringing the insecticide into contact with the parasite. A wide assortment of housing types and production systems are in use. One application method or system is seldom suitable for all types of housing. The type and form of parasiticide best suited for a

particular type of housing and management system should be determined and then used according to directions on the label. Fogging and misting can be effective only if the insecticide can be confined in the building and/or applied (blown) into cracks and crevices and on feathers where parasites are congregated. Otherwise, the effort and expense are largely wasted. Pyrethrum preparations containing synergists can be used in light- and temperature-controlled houses, but the automatic ventilation system must be bypassed and hand controlled during treatment. In high-rise houses, one must be sure to compensate for the large volume of space under the floors by using additional insecticide in a space-calculated application.

A common error is to assume that one application of insecticide will accomplish the objective. Parasite eggs are seldom destroyed; they remain to generate a new population, which must be attacked with a second application 2–3 weeks after the first. In addition, no system, application, or insecticide will result in a 100% parasite kill. After the parasite gains a foothold, it must be attacked repeatedly. Frequently, alternating insecticides and methods is necessary to effect control. Do not be misled by statements that the birds learn to live with their parasites. Such thinking encourages continuous poor bird performance and a host of problems.

Handling Precautions

Possible hazards to humans and animals from many of the modern pesticides must always be remembered when considering their use. It is best to wear a suitable mask, rubber gloves, and protective clothing when applying insecticides. The most important precaution in handling chemical insecticides is to read the directions, hazards, and antidotes on labels of containers before any use.

A basic rule in handling insecticides is to keep them properly labeled and stored in a locked building reserved for that purpose. Disposal of empty containers and discarded leftover insecticides is becoming more of a hazard and responsibility. Large drums should be returned to the supplier or heated to red heat for 5–10 minutes. Paper and plastic containers should be burned. Small glass and metal containers should be broken or punctured so that no one will use them for any purpose. In addition to the human hazard, discarded insecticides must not pollute lakes or streams nor become a hazard for honeybees. A safe policy is to check with the local Environmental Protection Agency (EPA) for recommendations.

Types

Crude Oil, Distillates, and Similar Compounds

Petroleum oils applied to clean buildings and equipment prior to introducing a new flock have been widely used to control lice, mites, and ticks. Oily residues get on the parasites and cause suffocation. They have been effective in getting at parasites in cracks and crevices of the building, but they cannot be applied to parasites on birds. Carbolinium, a wood preservative, also repels mites and other insects for long periods after it is applied. These products are quite messy and smelly and not as effective as many newer products.

Space Diffusion Insecticides

Pyrethrum products are released as a fog or mist, and the volatile compound permeates the room. Pyrethrum, an extract from plants, has low toxicity for higher forms of life but high toxicity for insects. It is relatively costly and may fail to penetrate adequately through feathers of birds and into insect hiding places. Synthetic forms of pyrethrum are now available commercially.

Vapona or DDVP, a preparation of dichlorvos, is sometimes impregnated into special materials from which it slowly vaporizes and diffuses through the air. This has greatest application in storage and other rooms that are closed and unventilated for long periods (overnight).

Systemic Inhibitors

Sulfaquinoxaline, used so extensively in feed and water to control coccidiosis and many bacterial infections, was found to rid birds of northern fowl mites (23). The product or its metabolites apparently create body conditions objectionable to the parasites (possibly odors), which drive them off the birds. The drug has since been banned from feed for hens laying eggs for human consumption, but other products have been reported to exert similar effects, and some may exert an unsuspected mite-repelling action. Drugs providing this type of mite control seem most effective when incorporated in feed prior to infestation and least effective as a treatment after infestation has become established.

Dusts and Sprays

Nearly all insecticides adaptable to control of parasites on fowl can be obtained as ready-to-use dusts or in the form of wettable powders, emulsifiable concentrates, or liquid suspensions, all of which can be prepared as sprays. Each has advantages, and suggested uses are supplied with the insecticide.

Chickens dust themselves instinctively. In litter-floor houses, insecticide dusts can be added to litter to control mites, according to specifications of the manufacturer. Special dust boxes with added insecticide can be placed in large cages and wire- or slat-floored houses to accomplish the same objective. Dusts can also be applied to birds in cages by using a dust applicator. The dust must be blown into the feathers to get at the parasites. Although laborious, dusting individual birds can be effective.

The most common method of applying insecticides is by spray. The mixture must be agitated during application to maintain a constant concentration and prevent separation. Sprays are mostly applied to floors and walls, but some can be sprayed on the birds.

None of the insecticides is perfect, and resistance is already known to have developed against some of them. New products are constantly being developed and tested for effectiveness. Poultry producers should be alert for products, preparations, and local vendors of professional pest-control services most suitable for their type of management system.

The best parasite control is to prevent the initial infestation through wise management practices. Once again, parasite infestations, like bacterial and viral diseases, can be most successfully controlled and eradicated from single-age farms or quarantinable units as part of a total, integrated system of "disease-prevention management."

Handling Disease Outbreaks

Observe the Normal

Good poultry producers watch feed and water consumption and egg production at all times, but more important, they observe normal sounds and actions of the flock. They sense immediately when any of these conditions are abnormal and interpret them as signs of abnormal health. When this happens, it should be assumed that an infectious disease has gained entry and may be tracked elsewhere during the investigation period. In a modern poultry production system, any disease creates serious disruption in the economical operation of the farm and the plants processing products from it. Serious infectious diseases can create havoc. The following steps should be followed when disease is suspected.

Look for Noninfectious Conditions

Take precautions against tracking an infectious disease that may be present, but investigate management errors immediately. A high percentage of so-called disease problems referred to laboratories for diagnosis are noninfectious conditions related to management: beak trimming errors; consumption of litter and trash; feed and water deprivation; chilling of chicks; injury from rough handling, automatic equipment, or drug injection; electrical failures; cannibalism; smothering; overcrowding; poor arrangement of feeders, waterers, and ventilators; inexpensive low-quality feed ingredients; ingredients causing feed refusal; improper particle size of feed ingredients; and rodent and predator attacks (1, 11). Zander observed a severe drop in egg production in a pathogen-free flock after a 48-hour failure of a mechanical feeder (77). Bell (10) observed marked reduction in lay from water deprivation related to a beak trimming system that resulted in long lower beaks, making it difficult to obtain water when the level was low. These are conditions that do not require services of a diagnostic laboratory. External parasites (mites, lice, and ticks) can be determined by producers if they examine affected birds.

Quarantine the Flock

In the event that no management factors can be found, the next step is to set up a quarantine of the pen, building, farm unit area, or entire farm, depending upon its design and programming. If this emergency was anticipated when the farm was laid out and programmed originally, the quarantine will be a minor problem. If the basic principle of "a single age in quarantinable units" was disregarded in original farm planning, a disease outbreak can be an economic disaster. Separate caretakers should be established for affected birds or at least sick ones should be visited last.

Submit Specimens or Call a Veterinarian

The owner or caretaker should submit typical specimens to a diagnostic laboratory or call a veterinarian to visit the farm and establish the diagnosis. Owners should seek professional diagnosis, rather than trying to hide some disease because of possible public recrimination. Veterinarians and caretakers can and should help dispel this apprehension by maintaining high ethical standards and refraining from discussing one producer's problems with others. Yet, there comes a time when all producers must be apprised of a problem. Service workers frequently are requested to examine the flock, select specimens for the laboratory, and initiate first-aid procedures until the veterinarian can be called or visited. If so, they should wear protective footwear and clothing when they enter the house. No other farm should be visited en route to the laboratory.

Diagnosis

It is important to get a diagnosis as soon as possible. The course of action will be determined by the nature of the disease. A producer should not procrastinate for any reason when a disease threatens, or it may get completely out of hand before a diagnosis is made. It is not always possible to treat a disease or check its deleterious effects, but to plan effectively for the future, it is important to identify any and all diseases that occur. A veterinarian should also be aware of the owner's economic plight at such times and render advice and assistance as quickly as information is available or a judgment can be made.

Special Precautions

In addition to causing serious losses in poultry, some diseases (chlamydiosis, erysipelas, and salmonellosis) are especially hazardous for humans. When these conditions are suspected or diagnosed, extra precautions must be taken to ensure against human infection. The proper government health authorities should be notified of chlamydiosis outbreaks, and all handling and processing personnel should be apprised of the disease, hazards, and necessary precautions.

In some states, certain diseases (*Mycoplasma* infections, avian chlamydiosis, and laryngotracheitis) must be reported immediately to the state animal disease control authorities so that proper investigation and action can be taken to protect the human population and the poultry industry. Common sense dictates that when a condition suggestive of an exotic disease, such as velogenic viscerotropic Newcastle disease, fowl typhoid, or avian influenza, is encountered, the proper state and federal regulatory authorities should be informed.

Nursing Care

Nursing care plays an important role in the outcome of a disease outbreak. Additional heat should be supplied to young chicks that begin huddling because of sickness. Clean and fresh (or medicated) water should be available at close range. Temporary, more accessibly located waterers are sometimes necessary during sickness. If water founts normally are located where chickens must jump onto some raised device or turkeys must cross through hot sunlight to reach them, the sick will not have the energy or initiative to seek water. They will soon become dehydrated, an early step on the road to death.

The same principles are true for feed. Sick birds can be encouraged to eat if the caretaker will proceed through the house, stirring feed and rattling feed hoppers or adding small quantities of fresh feed. Some antibiotics appear to stimulate feed consumption when included in the diet; however, any additive that proves distasteful to the bird should be removed immediately.

Sometimes birds become so depressed and moribund that the

caretaker must walk among them frequently to rouse them so that they will eat or drink.

Hopelessly sick and crippled birds should be killed in a manner to preclude or control the discharge of blood or exudates (see "Diagnostic Procedures"). Dead and destroyed birds should be disposed of immediately (see "Dead-Bird Disposal").

Drugs

No drugs should be given until a diagnosis is obtained or a veterinarian consulted. If the wrong drug is given, it can be a waste of money, or it may be harmful or even disastrous. If an infectious disease is found and corrective drugs are indicated, they should be used very carefully according to directions.

Strict regulations govern the use of drugs in mixed feeds for food-producing animals. For information, write to the U.S. Food and Drug Administration (FDA), 5600 Fishers Lane, Rockville, MD 20857. A handy reference is the annually updated *Feed Additive Compendium* published by Miller Publishing Co., Minnetonka, MN. Feed manufacturers must have FDA clearance to include drugs in mixed feeds. When treated flocks are to be marketed, a specified period (depending on the drug used) must follow cessation of treatment to allow dissipation of drug residues from tissues before slaughter. If the flock is producing table eggs when treated, the drug must be one permitted for use in laying flocks, or eggs must be discarded during, and for varying lengths of time after, treatment, which is a costly alternative.

If the flock is producing hatching eggs when it becomes infected and there is danger that egg transmission of the infectious agent from dams to offspring may occur (salmonellosis, mycoplasmosis, and avian encephalomyelitis), eggs should not be used for hatching until the danger has passed. It should also be kept in mind that in fertile eggs, residues of drugs used to treat breeders occasionally may cause abnormalities in some embryos.

Disposition of the Flock

The flock should not be moved or handled until it has recovered, unless the move is to a more favorable environment as part of the therapy. After treatment, if any, has been completed and the flock appears to be completely healthy, it may be marketed or moved to permanent quarters if such a move is part of the management program. Some healthy carriers may remain. If the flock is moved to another depopulated farm, this will present no problem except that occasionally a disease may flare up from stress of handling and moving. If the recovered flock is moved to a multiple-age farm, carriers can introduce the disease into susceptible flocks already there. If the recovered flock is already in permanent quarters having multiple ages, newly introduced flocks may be exposed and contract the disease, a common occurrence especially with respiratory and litter-borne diseases.

Diagnostic Procedures

Many satisfactory diagnostic and necropsy methods exist. The techniques and instruments used by one pathologist may vary considerably from those used by another. Some suggestions are offered here to guide the student and beginner. The goal of the necropsy is to determine the cause of impaired performance, signs, or mortality by examining tissues and organs, and to obtain the best specimens possible to carry out microbiologic, serologic, histopathologic, or animal inoculation tests. It is important that in the process, infectious materials do not endanger the health of humans, livestock, or other poultry. By proceeding in an orderly fashion, possible clues are less apt to be overlooked, and tissues will not be grossly contaminated prior to examination. Remember that a blood sample or tissue specimen determined later to be superfluous can always be discarded. It's better to save tissues and then discard them if they are later determined to be unnecessary or unimportant to the diagnosis.

A key to good poultry diagnosis is the art of "seeing the forest as well as the trees." Try to identify the most significant flock problem(s), rather than becoming engrossed in individual bird disorders. Watch for telltale patterns of pathology as presented by the total diagnostic consignment.

The techniques and procedures necessary to make an accurate diagnosis and identify specific disease agents are found in the technical information contained in succeeding chapters of this book and in the following excellent reference manuals: *A Laboratory Manual for Isolation and Identification of Avian Pathogens* (64), *Avian Disease Manual* (17), *Avian Histopathology* (53), and *Color Atlas of Diseases and Disorders of the Domestic Fowl and Turkey* (51). *Avian Hematology and Cytology* (15) should be consulted for detailed information on avian blood elements and methods for preparation and study. New information is continually being presented in the following journals, *Avian Diseases, Avian Pathology,* and *Poultry Science,* in the proceedings of several regional poultry disease conferences, and in other avian pathology and science journals.

Case History

The pathologist who has not seen the farm or the flock before attempting to diagnose the problem and recommend corrective measures is at a disadvantage. This can be partially overcome by getting a complete history of the disease and all pertinent events leading to the outbreak. The more information pathologists have about the history and environment, the more directly they can proceed to determining solutions for the problems. Unfortunately, the history includes only the situations, events, and signs that the caretaker, owner, service worker, or neighbor has observed and remembered. Knowledge of management factors such as ventilation; feeding and watering systems; accurate records of egg production, feed consumption, feed formulation, and body weight; lighting program; beak trimming practices; brooding and rearing procedures; routine medication and vaccination used; age; previous history of disease; farm location; and unusual weather or farm events may make the difference between diagnosis of the flock problem and the finding of a few miscellaneous conditions in a sample that may or may not be representative. Duration of the signs, the number of sick and dead, and when and where they were found dead can be important clues.

Poultry producers have developed a high degree of knowledge about poultry diseases and usually recognize those resulting in

dramatic or clear-cut signs and lesions. The veterinarian, therefore, is often confronted with obscure, undramatic, and complicated disease cases requiring extensive investigation. Even if all indications are that reduced performance is most likely due to a management factor, the veterinarian must check all reasonable disease possibilities. This requires a systematic approach to be sure that nothing is overlooked.

External Examination

Look for external parasites. Lice and northern fowl mites (*Ornithonyssus silviarum*) can be found on the affected chicken. If red mites (*Dermanyssus gallinae*) or blue bugs (*Argas persicus*) are suspected, examination of roosting areas and cracks and crevices in the houses and around the yards must be made, because these species do not stay on birds. See Chapter 26 for diagnosis and identification of external parasites.

The general attitude of live birds and all abnormal conditions should be noted carefully. It is very important to observe evidence of incoordination, tremors, paralytic conditions, abnormal gait and leg weakness, depression, blindness, and respiratory signs before the specimens are killed. It is very helpful to place birds in a cage where they can be observed after they have become accustomed to the surroundings and perform at their best. It is sometimes advisable to save some of the affected birds to observe possible recovery from a transitory condition (transient paralysis), respiratory infection, chemical toxicity, feed or water deprivation on the farm, or overheating during transport to the laboratory.

Examination should be made for tumors, abscesses, skin changes, beak condition, evidence of cannibalism, injuries, diarrhea, nasal and respiratory discharges, conjunctival exudates, feather and comb conditions, dehydration, and body condition. These are all useful clues.

Blood Samples

Blood specimens may be taken at this time (or immediately after the bird is euthanized). Frequently, it is desirable to have two (paired) blood samples several days apart to determine a rising or falling titer of antibodies to some disease (Newcastle disease) in the serum. In this case, a blood specimen may be taken from the main (brachial) wing vein or jugular vein or by heart puncture, and the bird then is saved for a second sample.

Venipuncture of the brachial vein is usually the simplest and best method for obtaining blood from turkeys, chickens, and most fowl under field conditions, especially when the bird is to be returned to the flock. Ducks are bled from the saphenous vein near the hock. Expose the vein to view by plucking a few feathers from the ventral surface of the humeral region of the wing. The vein will be seen lying in the depression between the biceps brachialis and triceps humeralis muscles. It is more easily seen if the skin is first dampened with 70% alcohol or other colorless disinfectant. To facilitate venipuncture, extend both wings dorsally by gripping them firmly together in the area of the wing web with the left hand. Insert the needle into the vein of the right wing holding the syringe in the right hand (Fig. 1.11). The needle should be inserted opposite to the direction of blood flow.



Fig. 1.11. Obtaining a blood sample from the wing vein.

Heart puncture can be made anteromedially between the sternum and metasternum (31), laterally through the rib cage, or anteroposteriorly through the thoracic inlet. Only through experience can one learn exactly where and at what angle to insert the needle. It is best to practice these techniques on freshly killed specimens before attempting to bleed live birds. A general rule for the lateral puncture is to form an imaginary vertical line at the anterior end of, and at a right angle with, the keel, and then palpate along that line. The heartbeat can be felt, and the needle inserted to the proper depth.

For heart puncture through the thoracic inlet, the bird should be held on its back with the keel up. The crop and contents are then pressed out of the way with a finger while the needle is guided along the ventral angle of the inlet. After penetrating the inlet, the needle is directed horizontally and posteriorly along the midline until reaching the heart.

The site for heart puncture between the sternum and metasternum is (in a mature chicken) about an inch above and posterior to the anterior point of the keel. The needle is directed at approximately a 45-degree angle in the anteromedial direction toward the opposite shoulder joint. The needle should pass through the angle formed by the sternum and metasternum and directly into the heart. For further details and illustrations, see Hofstad (31).

The size and length of the needle required for heart and venipuncture will depend on the size of the bird: for young chicks and poults, a 1/2-in. 20-gauge needle; for mature chick-

ens, a 2-in. 20-gauge needle. Mature turkeys may require larger needles. For quick and accurate bleeding, it is essential that the needle be sharp. A very slight vacuum should be developed intermittently to determine when vein or heart puncture has occurred. After vein puncture, a steady slight vacuum should be continuous to withdraw blood. If the vacuum is too great, the vessel wall may be drawn into the needle and plug the beveled opening. It is sometimes necessary to rotate the needle and syringe to be sure the beveled opening is free in the lumen of the vessel.

For most serologic studies, the serum from 2 mL blood is adequate. The blood should be removed aseptically and placed in a clean vial, which then is laid horizontally, or nearly so, until the blood clots. An occasional sample may require a long time to clot. This is especially true of turkey blood. Clotting can be hastened by adding a drop of tissue extract, made by killing and pooling a number of 10–12-day-old chicken embryos, grinding in a Waring blender, and freezing for future use. After the clot is firm, the vial may be returned to the vertical position to permit serum to collect in a pool at the bottom. Plastic vials are also available for blood collection. The clot does not adhere to the vial, and special positioning during clotting is unnecessary. Frequently, the serum from fat hens will appear milky due to lipids. Placing vials in an incubator will hasten the separation of the blood clot and serum. A fresh blood sample should never be refrigerated immediately after collection, as this will hinder the clotting process. Sera should not be frozen if agglutination tests are to be performed as this frequently causes false-positive reactions.

If an unclotted blood sample is required, it should be drawn into sodium citrate solution at the rate of 1.5 mL 2% solution/10 mL fresh blood, or deposited in a vial containing sodium citrate powder at the rate of 3 mg/1 mL whole blood, and the mixture should be gently shaken. One way to prepare tubes for collecting sterile citrated blood is to add the proper amount of 2% sodium citrate solution to the collecting tubes ahead of time and then sterilize the solution and evaporate the moisture in an oven.

Blood-collecting vials containing the anticoagulants heparin or EDTA can also be obtained commercially from laboratory supply companies. For certain types of serologic tests, fresh blood can be absorbed on the tips of filter paper strips, dried, and sent to the diagnostic laboratory, where antibodies can be recovered for testing by placing pieces of the treated paper into saline solution.

If a blood parasite or blood dyscrasia is suspected, smears of whole blood should be made on clean glass slides previously warmed to promote rapid drying. For staining techniques, see Campbell (15).

A drop of blood for a wet mount or smear may be obtained from very small chicks by pricking the vein on the posteromedial side of the leg or by pricking or cutting the immature comb.

Killing Birds for Necropsy

Cervical Dislocation

Several methods can be used to kill fowl, and each has certain advantages. The objective is to kill the bird instantaneously so it will not suffer in the process. Cervical dislocation, as described, is considered a humane method of poultry euthanasia by the American Veterinary Medical Association (AVMA) (5).

Bovine Burdizzo castration forceps can be used for killing large chickens and turkeys. It is difficult for one person to perform this operation and hold the bird at the same time, but it is quite easily done with the aid of an assistant. This technique also prevents agonal regurgitation and aspiration of crop contents into the respiratory passages if the forceps are left clamped until reflex muscle spasms cease. The neck of a young chick can also be broken easily by pressing it firmly against a sharp table edge, or by pinching between thumb and index finger, or by using the inside, noncutting angles of a surgical scissor such as a small Burdizzo.

Electrocution

Electrocution is a satisfactory method also. Clamps fixed to the end of electrical wires are fastened to the cloaca and mouth (this will assure moist contacts). The wires are then attached by means of a standard plug directly to 110-V alternating house current. A switch is thrown to feed the electric current through the wires. With this system, the bird rarely struggles and, thus, does not stir up dust or regurgitate crop contents. There is also less danger of agonal hemorrhages occurring or loss of blood when tissue specimens are desired. Obvious hazards to personnel and of short circuits on metal table tops should be recognized.

Other

Specimens selected for diagnosis may also be killed by intravenous injection of euthanasia solutions. Another method that would be satisfactory is euthanasia by placing the bird in a chamber filled with carbon dioxide (CO_2). Local availability of a CO_2 source may limit utilization of this technique.

Other methods of euthanasia can be found in a report of the AVMA (5). The method selected will depend upon the existing situation: species, size, and number of birds to be necropsied or sacrificed; tissues, fluids, and cultures to be taken; etc.

Necropsy Precautions

If there is reason to suspect that birds to be necropsied are infected with disease that may be contagious for humans (chlamydiosis, erysipelas, or equine encephalitis), stringent health precautions are essential. The carcass and the necropsy table surface should be wet thoroughly with a disinfectant. Good rubber gloves should be worn, and care should be taken that neither the pathologist nor assistants puncture the skin of their hands or inhale dust or aerosols from tissues or feces. It is advisable to wear a fine-particle respiratory mask to prevent inhalation of contaminated dust. All laboratory personnel who may come in contact with carcasses, tissues, or cultures should be informed of their possible infectious nature and precautions to be taken.

With some notable exceptions (see sections on the specific diseases), most commonly encountered poultry disease agents are not considered pathogenic for humans. Nevertheless, it is wise to wear rubber gloves at all times while performing necropsies. For a review of poultry diseases in public health, see Galton and Arnstein (25). Adequate instruments for routine work are necropsy shears to cut bones, enterotome scissors to incise the gut, a necropsy knife to cut skin and muscle, and a scalpel for

fine examination of tissues. These should be supplemented with forceps, sterile syringes, needles, vials, and petri dishes for collecting blood samples and tissue specimens as the situation dictates.

Necropsy Technique

Internal Organs

The specimen is laid on its back and each leg in turn drawn outward away from the body while the skin is incised between the leg and abdomen. Each leg is then grasped firmly in the area of the femur and bent forward, downward, and outward until the head of the femur is broken free of the acetabular attachment so that the leg will lie flat on the table (Fig. 1.12A).

The skin is cut between the two previous incisions at a point midway between keel and vent. The cut edge is then forcibly reflected forward, cutting as necessary, until the entire ventral aspect of the body, including the neck, is exposed (Fig. 1.12B). Hemorrhages of the musculature, if present, can be detected at this stage.

Either of two procedures is now used to expose the viscera. The poultry shears are used to cut through the abdominal wall transversely midway between keel and vent and then through breast muscles on each side (Fig. 1.12C). Bone shears are used to cut the rib cage and then the coracoid and clavicle on both sides (Fig. 1.12D). With some care, this can be done without severing the large blood vessels. The process may also be done equally well in reverse order, cutting through the clavicle and coracoid and then through the rib cage and abdominal wall on each side. The sternum and attached structures can now be removed from the body and laid aside. The organs are now in full view and may be removed as they are examined (Fig. 1.12E,F).

If a blood sample has not previously been taken and the bird was killed just prior to necropsy, a sample can be promptly taken by heart puncture before clotting occurs. Large veins leading into the leg may be incised, allowing blood to pool in the inguinal region for subsequent collection.

Laboratory Procedures

Bacterial Cultures

If gross lesions indicate bacterial cultures are needed, they can be made from unexposed surfaces of the viscera without searing the surface. If contamination has occurred, the surface of the organs should be seared with a hot spatula or other iron designed for that purpose before inserting a sterile culture loop. Care must be taken not to sear and heat the tissue excessively. It is often desirable to transfer large tissue samples aseptically to a sterile petri dish and take them to the microbiology laboratory for initial culture in cleaner surroundings.

Respiratory Virus Isolation

If a respiratory disease is suspected and virus culture or bird passage is desirable, an intact section of lower trachea, the bronchi, and upper portions of the lungs is removed aseptically with sterile scissors and forceps and transferred to a sterile container. Other tissues (air sac tissue) can be added aseptically to the sample or transferred to other sterile containers for separate study. The trachea can now be incised; if exudate is present, it can be added to the preceding collection or saved in separate vials. Similar procedures can be followed for initial virus isolation from various parenchymatous organs.

Salmonella Cultures

All other visceral organs should be examined for abnormalities (microabscesses, discoloration, swelling, and friability). If abnormalities are observed, inoculum from the affected tissues should be transferred to suitable solid or liquid media for culture before the intestinal tract is opened. Once opened, gross contamination of other organs with gut contents is almost certain to occur. If *Salmonella* infection is suspected, selected sections of the gut are removed with sterile forceps and scissors and placed directly into a sterile petri dish for later culture. For routine examination, a single section comprising the lower ileum, proximal portions of the ceca and cecal "tonsils," and proximal portion of the large intestine may be used. All are minced or ground aseptically to produce an inoculum. Additional areas of the intestinal tract or tissues of other visceral organs may be added to the gut collection or cultured separately. Alternatively, sterile swabs may be used to obtain samples from the exposed gut lining for *Salmonella* cultures. See Chapter 2 of *A Laboratory Manual for Isolation and Identification of Avian Pathogens* (64) for detailed culture technique.

Gross Necropsy

After necessary cultures have been collected, a thorough gross examination of all tissues should be performed. Enlargement of the liver, spleen, and kidney should be evaluated. A clear indication of hepatomegaly is rounded liver margins. The intestine may be examined for inflammation, exudates, parasites, foreign bodies, malfunctions, tumors, and abscesses. The various nerves, bone structure, marrow condition, and joints can now be examined. The sciatic nerve can be examined by dissecting away the musculature on the medial side of the thigh. Inside the body cavity, the sciatic plexus is obscured by kidney tissue. These nerves can best be exposed by scraping away the tissue with the blunt end of a scalpel. Nerves of the brachial plexuses are easily found on either side near the thoracic inlet and should be examined for enlargement. Examination of vagus nerves in their entirety should be made; otherwise, short enlargements may be missed.

The ease or difficulty with which bones can be cut with the bone shears is indicative of their condition. The costochondral junctions should be palpated and examined for enlargement ("beading") and the long bones cut longitudinally through the epiphysis to examine for abnormal calcification. Rigidity of the tibiotarsus or metatarsus should be tested by bending and breaking to check for nutritional deficiency. A healthy bone will make an audible snap when it breaks. Bones from a chicken deficient in vitamin D or minerals may be so lacking in mineral elements that they can be bent at any angle without breaking.

Joint exudate, if present, can be removed after first plucking the feathers and searing the overlying skin with a hot iron. After searing, the skin may be incised with a sterile scalpel and exudate removed with a sterile inoculating loop or swab. Paranasal sinus exudates can be removed and examined in a similar manner.

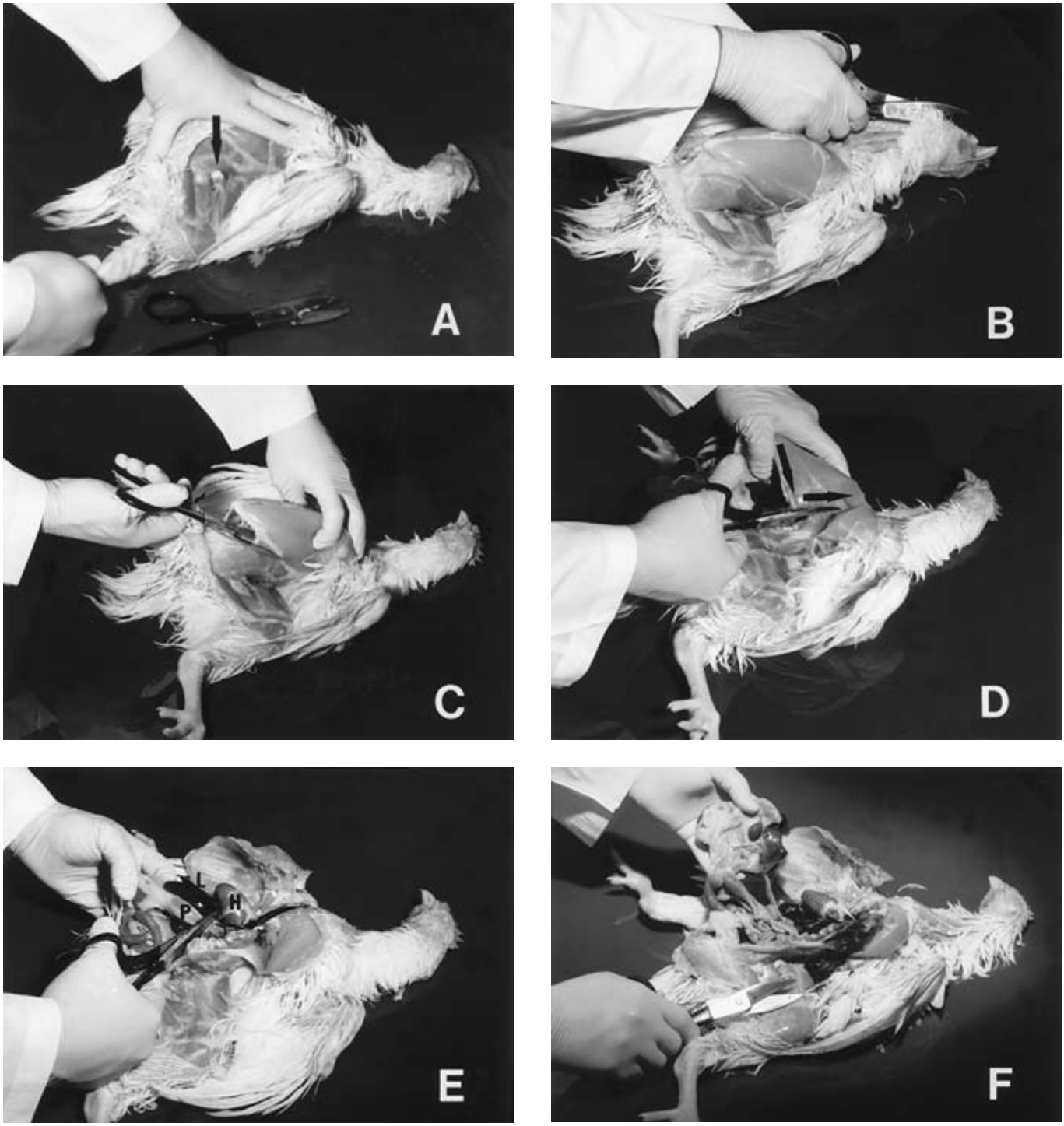


Fig. 1.12. Each pathologist will develop his/her own systematic technique for conducting a necropsy. A sturdy pair of poultry shears is usually sufficient to conduct a necropsy. Other instruments such as scissors, forceps, and scalpel may be helpful in collecting small or delicate samples. A knife may be needed to cut through joints and bone. The illustrated technique will aid the beginner. A. The skin and fascia between the leg and abdomen are cut, and the legs are pulled and twisted to disarticulate the head of the femur (arrow) from the hip. B. The skin from the vent to the beak is incised and reflected. C. The body cavity is entered at the ventral tip of the sternum. The incision is made at the margin of the pectoral muscle and continues through 2–3 ribs. A similar incision is made on the opposite side of the breast. D. The shears are reoriented (arrows), and the incision is continued through bone and muscle to the thoracic inlet. The breast is broken over to the opposite side (or removed) exposing the viscera. At this point of the necropsy, microbiological samples are collected. E. The intestinal viscera are freed by cutting through the esophagus and vessels of the liver just anterior to the proventriculus and liver. Heart (H), liver (L), and proventriculus (P) are indicated. F. The intestines can be removed by gentle traction, which tears mesenteric and air sac attachments. The lungs, heart, and kidneys remain in the body cavity for later examination.

Exposure and Removal of Brain

Removing the intact brain is not easy, since meningeal layers are attached firmly to bony structures in some places. The following technique can be performed quickly and is satisfactory for examination and removal of the brain in most instances.

Remove the head at the atlanto-occipital junction and remove the lower mandible. Sear the cut surface and trim away excess loose tissue. Reflect the skin forward over the skull and upper mandible and hold it firmly in that position with one hand. Sterile instruments should be used for the succeeding steps if a portion of the brain is desired for animal inoculation, virus isolation, or fungal or bacterial culture.

With the sterilized tips of heavy-jawed bone shears or strong surgical scissors, nip just through the bone to the cranial cavity on both sides of the head, beginning at the occipital foramen and proceeding forward laterally to the midpoint at the anterior edge of the cranial cavity (Fig. 1.13A). Lift off the cut portion of bone and expose the entire brain (Fig. 1.13B).

If a portion is needed for culture or animal inoculation (e.g., avian encephalomyelitis virus suspect) and also one for histopathologic examination (e.g., vitamin E deficiency), cut the brain medially from anterior to posterior along the midline with a sharp, sterile scalpel blade. With sterile, sharp curved scissors, cut the nerves and attachments carefully from one of the brain halves while the head is tipped upside down, so that the loosened portion falls into a jar of formalin as it is freed (Fig. 1.13C). The second half can now be removed aseptically (but without concern for preservation of tissue structure) to a sterile petri dish or sterile mortar and pestle. Be careful not to contaminate brain tissue intended for virus isolation with instruments that have been in contact with formalin. The separate halves may also be removed in reverse order (Fig. 1.13D). If all of the brain is required for either purpose, proceed with proper precautions for the purpose intended. If the brain is destined only for sectioning, it may be fixed *in situ* and then removed. Large brain portions should be incised longitudinally to permit good penetration of fixative.

Tissues for Histopathologic Examination

Frequently, stained tissue sections are needed. The quality of the slide is no better than the quality of the specimen and the care taken to preserve it. For good preservation, the tissue pieces from killed birds should be saved immediately after death, especially brain and kidney tissues, which deteriorate rapidly. Specimens should be small to allow quick penetration of fixative, gently incised with a sharp scalpel or razor blade to preserve tissue structure, and preserved in 10 times their own volume of 10% formalin or other fixative. Bone pieces should be sawed with a sharp bone saw unless thin or soft enough to cut with scissors or scalpel. After proper labeling and dating, they should be sent immediately to the processing laboratory.

Lung tissue usually floats on the surface of the fixing solution because of trapped air. Satisfactory fixation can be accomplished by placing absorbent cotton over the tissue, which serves to keep it immersed. Methods to exhaust air from air spaces in lung tissue by creating a vacuum over the fixative can be used but are less satisfactory and may result in artifacts.

After fixing, bone tissue must be decalcified by immersion in a decalcification solution made by mixing equal parts of aqueous 8% hydrochloric acid and aqueous 8% formic acid (50). Decalcification typically takes 1–3 days, the length of time depending on the size and density of the bone sample.

If eye tissue is to be saved for sectioning, the whole eye should be removed and all ocular muscles trimmed off the globe to allow for rapid penetration by the fixative.

Any tissue held too long in formalin fixative becomes excessively hard. If processing is to be delayed, tissues should be transferred to 70% alcohol after 48 hours in fixative. Textbooks on histologic techniques (49, 50, 65) should be consulted for detailed procedures.

Progressive Examination Hints

The following procedures during the course of necropsy may be helpful to the beginner in checking for some commonly encountered diseases. They are not intended as definitive diagnostic methods. To arrive at a diagnosis, the student and beginning diagnostician must refer to the characteristic signs and lesions, diagnostic procedures, and characteristics of the infectious agent discussed under the specific diseases in succeeding chapters, and also to the manual, *Isolation and Identification of Avian Pathogens* (64).

Coccidia. Observe and note the subserosa before incising the intestine. Make wet mount smears of mucosal scrapings from various segments of the intestine and cecal contents and examine directly under the microscope for suspended oocysts and merozoites and stages undergoing development in epithelial cells (tissue stages).

Other Protozoa. Make wet mounts of affected areas, adding a little warm physiologic saline solution if necessary to provide fluid, and examine under a microscope for hexamita, histomonads, and trichomonads.

Capillarids and Ascarid Larvae. Collect mucous exudate and deep mucosal scrapings and press into a thin layer between two thick pieces of plate glass. Examine before a strong light or under low-power magnification for the presence of parasites. Under magnification, look for bi-polar, lemon-shaped eggs in the female capillarids.

Fungi. Make wet mount smears of scrapings of affected areas and add 20% sodium or potassium hydroxide. Digest with frequent warming for 15 minutes or more and examine under high-power magnification for mold hyphae.

Campylobacter. Examine fresh bile wet mounts under dark-field or phase illumination. Only positive findings may have significance.

Bacteremia and Blood Parasites. Make fresh mounts, preferably with citrated blood, and examine under light- and dark-field illumination for viable organisms. Make fresh blood smears

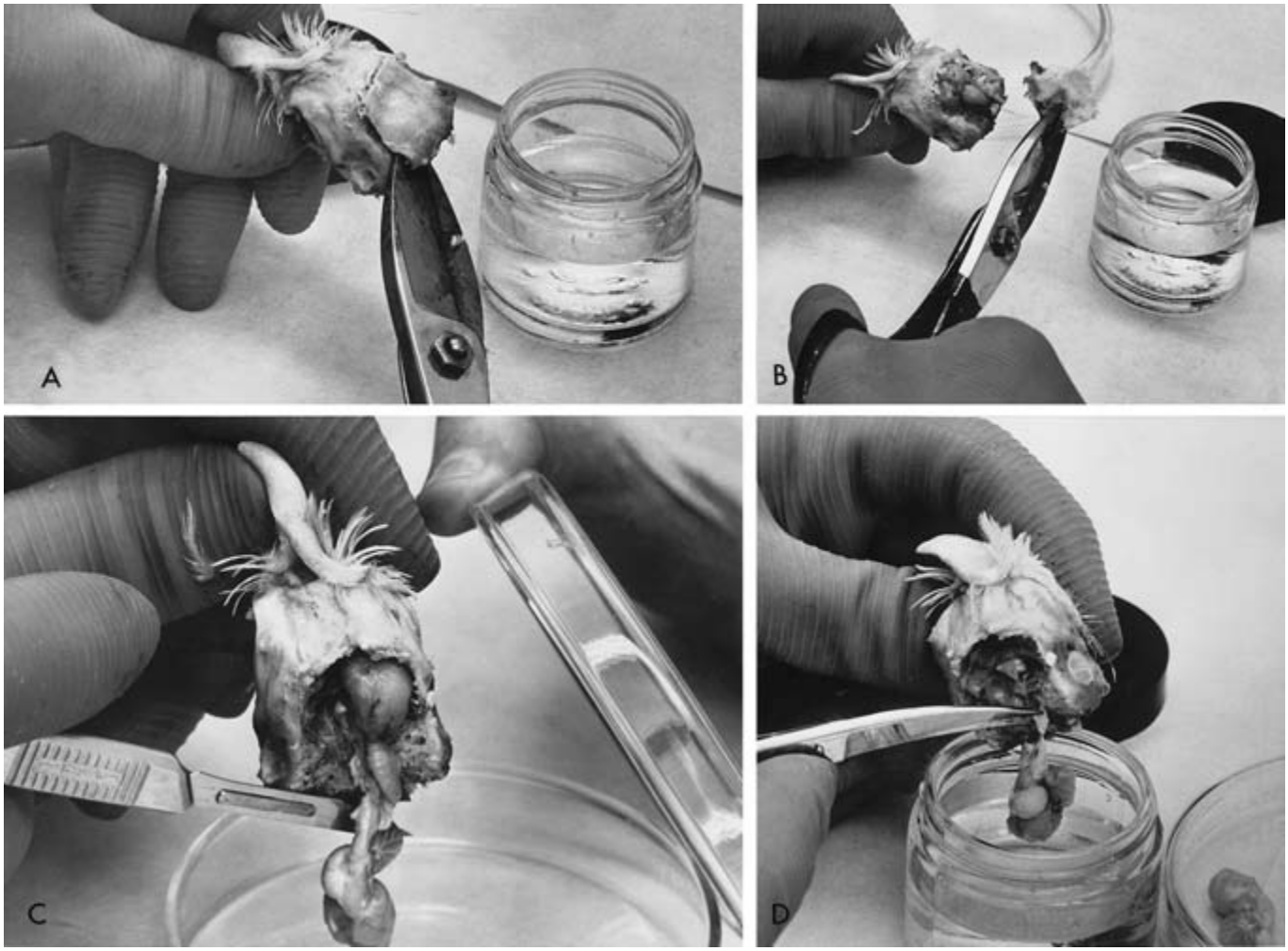


Fig. 1.13. With a little practice, the brain can be removed with a minimum of trauma. A. Incise bone all the way around the periphery of the cranial cavity with heavy bone shears. B. Remove loosened portion of the bony skull. C. Incise brain longitudinally with sterile, sharp scalpel and remove one-half for sterile culture technique. D. Remove second half by dropping it into 10% formalin for histologic techniques.

and air dry for staining by Giemsa's, Gram's, Wright's, or other method.

Exudates. If infectious coryza is suspected, make thin smears of clear nasal or sinus exudate for staining by Giemsa's, Gram's, methylene blue, or other method. Inoculate appropriate media or susceptible chickens for isolation of the organism.

Abscesses. Select appropriate culture media suitable for the growth of a variety of infectious organisms that may be suspected of causing the abscess. Sear and incise the surface of the abscess and inoculate culture media with the extracted material, using a sterile inoculating loop or swab. Make smears from the abscess on clean glass slides, diluting with a drop of water if the material is too thick. Air dry and flame slides and make Gram's, acid-fast, or any other stain as desired.

Embryo Inoculation for Virus Isolation. For routine virus isolation, centrifuged and/or filtered fine-ground suspensions of suspect tissues (trachea, bronchi, lung, liver, spleen, kidney, brain, bone marrow) or body fluids and exudates may be inoculated into the chorioallantoic cavity and yolk sac and onto the chorioallantoic membrane (CAM) of embryos at various stages of incubation. See discussions of the specific diseases for virus culture techniques. Also see *A Laboratory Manual for the Isolation and Identification of Avian Pathogens* (64) for selection of the proper age of embryo and route of inoculation for various disease agents as well as detailed inoculation procedures. Embryos from specific-pathogen-free dams should be used for culture to be sure that any agent recovered originated in the inoculum and not in the dams that produced the eggs. Equally important is assurance that negative cultures are due to absence of infectious agents in inoculum, rather than to interference of passive antibodies in eggs. Because the purpose of virus isolation is to determine which may be present, it is advisable to inoculate various ages of embryo by the various routes. Several blind passages may be necessary before the culture attempt can reasonably be considered negative. A simple technique that does not require dropping the CAM has been described (29).

The CAM may be drawn away from the shell (dropped) to facilitate inoculation. First, drill or punch a small hole in the shell over the air cell and then slowly drill or punch a second hole through the shell at a point on the side over the embryo. Applying mild suction through a rubber tube over the hole into the air cell causes the CAM to drop away from the inner shell membrane under the second drilled hole. A bright candling light should be used while suction is applied to determine when the CAM has dropped.

For yolk sac inoculation, the needle can be directed through the air cell and directly to the center of the egg. Some yolk may be withdrawn into the syringe to verify the location of the needle.

For chorioallantoic cavity inoculation, a hole is drilled over the edge of the air cell at a spot previously marked with the aid of a candling light. The cavity lies adjacent to the shell and can be easily penetrated from that point. All holes should be sealed with suitable sterile material before reincubating.

Cell culture procedures are becoming more common in diagnostic laboratories. Technicians with this capability may inoculate the cell cultures directly with tissue extracts or body fluids, or they may use embryos for primary screening and transfer embryo fluids or extracts to cell culture for further study and identification.

Disposing of the Specimen

If a disease infectious for humans is suspected, the carcass should be autoclaved, incinerated, or otherwise rendered incapable of causing infection to laboratory or other personnel. Similar precautions should be followed during disposal of carcasses infected with a virulent poultry pathogen that presents a health hazard to the industry. The necropsy area, instruments, and gloves should then be cleaned, washed, and disinfected.

Communication

Flock owners are not always interested in technical data. They want to know what the problem is and what should be done to correct it and/or how to prevent reoccurrences. Sometimes technical data are necessary to clarify the diagnosis, but the report should be in language and terms that they will understand. A minimum of complicated scientific and medical technology words should be used. When medical terms are apt to be confusing, they should always be explained in lay terms.

The report should include the necropsy findings, results of laboratory studies, (histopathologic, serologic, and cultural), diagnosis (temporary or final), and conclusions and recommendations. The owner is seeking professional advice. The veterinarian should give his/her best conclusions and recommendations based on the facts available. A verbal report or telephone call to the flock owner, manager, or service worker soon after completion of the necropsy and initial tests is highly advisable. A tentative diagnosis can be offered pending further confirmation.

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Antimicrobial Therapy

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Introduction

Even though the trend in the poultry industry is toward preventative disease programs and improved management practices, the occurrence of bacterial diseases is not uncommon. Medicating poultry has been performed by the industry for many years and will continue to play a role in successful commercial production. Because of a limited spectrum of antibiotics available in the United States, we must ensure that when we do treat, we use information and procedures that best place us in a position to be as successful as possible. When initiating medication use, we must consider all aspects of therapy to ensure the best possible outcome.

The key to antibiotic treatment success is related to many principles and includes identification of the pathogen; basing antibiotic selection on sensitivity results; effective antibiotic concentrations present at the infection site; proper dosing and route of administration; and responding to management needs. Antibiotic therapy should be used as a tool to manage disease outbreaks and not to be used as a crutch for deficiencies in management or nutrition. Because many of the diseases that occur in poultry are secondary to other primary infections and conditions, identifying the primary cause of infection is paramount in modern poultry production to minimize any overuse of antibiotics.

Overall, specifics regarding antibiotic therapy in poultry in the literature are lacking. Much of the discussion in this chapter is based on the clinical experience of the author. In this chapter, routes of medication, properties of antibiotics, and general antibiotic use considerations are discussed. General principles established and supported by the American Veterinary Medical Association regarding judicious therapeutic antimicrobial use are also listed for reference in antibiotic use protocols.

Routes of Medication

Three routes exist for administering antibiotics in poultry: injection, water, and feed. By far, the most common treatment route is by water. It provides rapid blood levels in diseased birds and is administered easily for mass application. Water therapeutic intervention can be performed for a minimum of 3 and up to 7 days. Premature withdrawal or change in therapeutic water medication should be done based on only clinical assessment, diagnostic sampling results, and/or a 2–3 fold rise in mortality. Feed grade antibiotics are used therapeutically on a limited basis. Feed grade antibiotic therapy provides excellent follow-up therapy after an initial water treatment and can be used for long-term therapy. Problems related to feed grade antibiotic use include delayed therapeutic blood levels, and diseased birds may have decreased feed consumption. In the United States, feed grade antibiotics can be used only in accordance with the Feed Additive Compendium, published in cooperation with the Animal Health Institute (2). Extra label antibiotic use in feed is prohibited under the Animal Medicinal Drug Use Clarification Act of 1996 (AMDUCA)(1). Feed grade antibiotics are more commonly used in poultry for disease prevention, such as necrotic enteritis control. Injectable antibiotics occasionally are used in breeders in an extra label manner, but more commonly are used either *in ovo* or injected at one day of age for bacterial omphalitis control. Any extra label use of injectable antibiotics is defined and covered under AMDUCA (1).

Antibiotic Considerations

Historically, poultry have been treated through the water based on a volumetric approach (i.e., based on a known concentration of antibiotic in the drinking water, ppm or mg/gal). This volumetric

dosing is generally described on product labeling. Volumetric dosing is performed by mixing a stock solution of concentrated antibiotic at the manufacturer's recommended instruction and delivering the antibiotic through a proportioner at the rate of 1 ounce of the antibiotic stock solution per gallon of drinking water. This dosing regimen has been in place throughout the world for years. However, any increase in water consumption will, increase the cost of medication and potentially result in residues and/or toxicity. Likewise, decreased water consumption and subsequent decrease in antibiotic uptake usually are interpreted as lack of antibiotic efficacy. Most other animals, including those used for food, are treated based on body weight. No present evidence suggests that this method of treatment would not be successful in poultry. Except for the tetracycline class of antibiotics (dosed at 55mg/kg), mg/kg dosing is not well documented with antibiotics for poultry. The inconsistency in water consumption makes it imperative that we look at more scientific approaches to antibiotic treatment in poultry. We must realize, however, with current regulatory issues in place, most dosing based on body weight is considered extra label and requires a veterinary prescription in the United States (1). Also, antibiotic withdrawal times are based on labeled indications, which may not be based on mg/kg of body weight. Therefore, if dosages other than labeled recommendations are used, an extended withdrawal time may be necessary.

Water consumption is affected by water quality, which includes mineral content, pH, and nitrate content. Increased combinations of magnesium, sulfates, and chlorides in water can produce laxative effects and increase water consumption. It is well recognized in the poultry industry that high sodium/salt levels will increase water consumption (5). When the pH of water falls below 4, water consumption tends to decrease. Poor weight gain associated with high nitrate levels above 50 ppm has been associated with decreased water consumption and overall poor performance (5).

Feed ingredients such as protein sources, sodium/salt content, and energy density also affect water consumption. Fish meals, bakery by-product meals, and certain phosphorous sources can tend to increase water consumption based on sodium and/or salt content or the presence of biogenic ammines. Different protein sources and shipments can be inconsistent in their nutritional analysis and result in variations in water consumption. Water consumption is also impacted by environmental temperature. Most water consumption charts are based on an environmental temperature of 70°F. For every 1° increase in environmental temperature, a corresponding increase occurs in water consumption of approximately 4%. Therefore, for every 5-degree increase in temperature, a corresponding 20% increase in water consumption can be anticipated. However, when environmental temperatures are elevated above 90°F, overall activity of the birds tends to decrease and water consumption may be impacted.

All of the preceding information supports the conclusion that birds might best be treated based on body weight and not on water consumption alone.

Antibiotics and Antibiotic Properties

Each antibiotic possesses properties that allow it to have advantages and/or disadvantages compared to other antibiotics. These properties include their spectrum of activity and mechanism of activity. A bactericidal drug kills the bacteria; whereas a bacteriostatic drug inhibits the replication of the bacteria and requires a functional immune system (defense mechanism) to eliminate the bacteria from the body. Hence, a bacteriostatic antibiotic may have limited value in a chronic infection because of the duration of the illness and impairment of the body's defense mechanisms. A bactericidal product can be used in acute and/or chronic infections. Antibiotics are either broad in their spectrum of activity or narrow. That is, they may have an antibacterial spectrum against either gram-positive bacteria, Gram-negative bacteria, or bacteria in both classes. As a rule of thumb, most broad-spectrum antibiotics are bacteriostatic, and many of the narrow spectrum antibiotics are usually bactericidal. Exceptions to this rule include high doses of erythromycin and the tetracyclines, which are broad in spectrum and bactericidal in their action.

A limited number of antibiotics are cleared for use in poultry. Any antibiotic not specifically labeled for use in the species for the disease in question (indication) should be used by prescription only and be recognized as an extra label drug use. In the United States, this extra label drug use is also defined under the AMDUCA (1). Extra label drug use is permitted only when prescribing antibiotics in water and as injections. It does not allow the addition of antibiotics, with or without a prescription, in feed if not approved as listed in the *Feed Additive Compendium* (2). Likewise, illegal drugs (chloramphenicol, nitrofurazone, nitroimidazoles, etc.) cannot be used in an extra label manner. The following drugs are approved for use in poultry the United States. Action, spectrum, and approved route of administration are presented in Table 1.4.

Mixing Antibiotics (Compounding)

Many times, when faced with an acute disease outbreak, the combination of antibiotics is considered to broaden the scope of antibacterial activity. Mixing antibiotics, which are not approved for use in combination, constitutes a new animal drug in which noninterference, safety, and residue studies have not been performed and/or approved by the Food and Drug Administration. At this time, this policy deals only with antibacterial agents and does not apply to vitamins, minerals, and electrolytes. Mixing antibiotics not demonstrated to be safe and effective could potentially change the amount of individual drug absorbed and, thus, alter the excretion rate of the drug from the body (6). This excretion rate change may result in toxicity or potential illegal drug residues. Likewise, mixing drugs may affect the absorption to the extent that efficacy is negatively impacted.

Antibiotics that are weak acids should not be mixed with weak bases. Weak acids include the sulfonamides and penicillin. Weak bases include erythromycin, streptomycin, gentamicin, neomycin, tetracyclines, and lincomycin. Antibiotics shown to be more effective in basic solutions (>pH 7) include the sulfonamides and penicillin (6). Erythromycin and tetracyclines are more effective in acidic solutions (pH 6–7) (6). Specific interactions of antibiotics used in poultry have been reported (6). The addition of vitamins and electrolytes may affect the pH of antibiotic stock solutions. Penicillin should not be mixed with vitamin preparations.

Table 1.4. Action, spectrum, and approved route of antimicrobial administration in poultry[A].

Antibiotic	Action	Spectrum	Route of Administration
Bacitracin	Cidal	Gram +	Feed/water
Ceftiofur	Cidal	Gram + & –	Injection
Chlortetracycline	Static	Gram + & –	Feed/water
Oxytetracycline	Static	Gram + & –	Feed/water
Tetracycline	Static	Gram + & –	Water
Erythromycin	Static	Gram + & –	Feed/water
Gentamicin	Cidal	Gram + & –	Injection
Lincomycin	Static	Gram + & –	Feed/water
Lincomycin/spectinomycin	Static	Gram + & –	Water
Neomycin	Cidal	Gram –	Feed/water
Neomycin/oxytetracycline	Cidal	Gram + & –	Feed
Novobiocin	Cidal	Gram +	Feed
Penicillin	Cidal	Gram +	Feed/water
Spectinomycin	Static	Gram + & –	Water/injection
Streptomycin	Cidal	Gram –	Water
Sulfadimethoxine	Static	Gram + & –	Water
Sulfadimethoxine/ormetoprim 5:1	Static	Gram + & –	Feed
Sulfaquinoxaline	Static	Gram + & –	Water
Tylosin	Static	Gram + & –	Feed/water
Virginiamycin	Static	Gram +	Feed
Bambermycin[B]	N/A	N/A	Feed

[A]Data included is an accurate reflection of approved antimicrobials in the United States at the time of publication.
[B]Bambermycin is a feed additive having no specific antibacterial action.

Chlortetracycline and penicillin have been shown to be antagonistic in combination.

Treatment Records

With any treatment regimen, accurate records should be maintained. An important part of this record system is to collect bacterial cultures and conduct antimicrobial susceptibility testing. This data will ensure that therapy decisions are correctly directed and will provide a baseline for the recognition of the development of antimicrobial resistance in the future. Furthermore, a record of all antibiotic successes and failures will help to guide future antibiotic therapy decisions.

Antibiotic Resistance

Interest and concern have increased regarding the administration of antibiotics in food-producing animals and its emergence of populations of bacteria that are resistant to currently available antibiotics. From the poultry health perspective, the concern is that poultry pathogens will develop resistance to currently approved antibiotics. From the human health perspective, there is concern that food-borne bacteria that cause illness in humans will be resistant to antibiotic treatment or that food-borne bacteria will transfer resistance to human commensal or pathogenic bacteria and that these organisms will likewise be refractory to treatment. Both concerns are real and should not be minimized as classes of antibiotics that are used in poultry are also used in humans. Research is needed in this area to determine whether the use of antibiotics in poultry has played a role in the development of antibiotic resistance in humans. All parties involved in using antibiotics should be aware of this concern and use antibiotics as judiciously as possible. The bacteria of concern from a human health standpoint are *Campylobacter* spp., *Salmonella* spp., and *Enterococcus* spp. None of the previously named bacteria are of significant clinical concern in poultry flocks (i.e., very few clinical diseases are caused by them). However, we must be aware that any time antibiotic treatment is performed, these organisms are exposed to the antibiotic, and resistance can develop as a consequence of this exposure.

Antibiotic resistance is a natural phenomenon that occurs when bacteria are exposed to antibiotics but also occurs without antibiotic exposure in the case of inherent resistance (4). The use of antibiotics in animals and humans has accelerated the rate of resistance development by increasing selection pressure placed against both pathogenic and nonpathogenic bacteria. We must remember that when poultry are treated with antibiotics, it affects both target and nontarget bacteria, and resistance in both classes of bacteria may develop. Resistance development occurs by the exchange of extra-chromosomal DNA called plasmids and through genetic or chromosomal changes within the bacteria. Plasmid-mediated resistance can occur within or between the same or different populations of bacteria (4).

Two basic methods are used in determining the susceptibility of bacteria to antimicrobials: disk diffusion and dilution (7). The disk diffusion method is most commonly used in diagnostic laboratories. The dilution method commonly is used so that the antimicrobial concentration can be diluted serially to provide mini-

mum inhibitory concentrations (MIC) for individual organisms against antimicrobials tested. Both disk diffusion and dilution methods are *in vitro* procedures performed in the laboratory. Host interactions, drug pharmacokinetics, and other practical aspects of antimicrobial therapy must be considered for the veterinarian to predict the *in vivo* or animal response to a particular disease antimicrobial therapy. Both methods should be standardized for reproducibility and confidence in the results (7).

Because antibiotic resistance is one consequence of antibiotic use, we must ensure the judicious use of antibiotics in poultry. National and international organizations such as the American Veterinary Medical Association (AVMA) and World Health Organization (WHO) have supported judicious therapeutic antimicrobial (antibiotic) principles for use in all animals, including food animals such as poultry. These principles, presented in the following section, are approved by the AVMA and have been applied to poultry to be used as guidelines to optimize therapeutic efficacy while minimizing the development of resistance to protect both animal and public health. After each principle is listed, a discussion of its importance in poultry follows.

Judicious Use Principles for Poultry

The principles enumerated here should be incorporated into the thought process when the decision is made to use therapeutic antimicrobials in our poultry flocks (3).

1. Preventive strategies, such as appropriate husbandry and hygiene, routine health monitoring, and immunization, should be emphasized.

   Minimizing antimicrobial use through disease prevention is the fundamental principle that has historically led to the success of poultry companies. Farms using all-in-all-out production minimize the presence of multiple ages of flocks on farms to help in disease prevention. Biosecurity programs in place on poultry farms prevent the introduction of diseases. The use of coveralls, boots, and head coverings can prevent the introduction and spread of disease within and between farms. Preventative disease programs based on vaccination strategies reduce disease outbreaks in poultry. The poultry industry is the leader in novel vaccination procedures for vaccination of large numbers of animals. Breeder and meat production flocks are monitored for protective response to vaccinations. Serological monitoring of disease exposure forms the basis of our strategic vaccination programs.
2. Other therapeutic options should be considered prior to antimicrobial therapy.

   The poultry industry approaches the treatment of diseases with antimicrobial agents very seriously. Because of the cost of disease treatment with antimicrobials, therapeutic antimicrobial intervention should be used only as a tool to treat active disease. Management adjustments should be made when disease outbreaks occur by reacting to environmental temperature, ventilation, and litter moisture to minimize the impact of any disease condition in flocks. Supportive therapy with vitamins and electrolytes can be used in some cases of disease outbreaks. All of the preceding strategies should help in preventing the use of antimicrobials for treatment.
3. Judicious use of antimicrobials, when under the direction of a veterinarian, should meet all requirements of a valid veterinarian-client-patient relationship.

   Poultry veterinarians, in integrated companies, should closely monitor antimicrobial use in their poultry flocks. They should maintain close contact with service technicians and managers related to the use of antimicrobials. Veterinarians are involved in the training of all individuals who will ultimately be following veterinary directions for antimicrobial use. Antimicrobials should be used always under the direction and knowledge of the company veterinarian or veterinary consultant.
4. Prescription, Veterinary Feed Directive, and extra label use of antimicrobials must meet all the requirements of a valid veterinarian-client-patient relationship.

   At the present time, no feed additives are approved for prescription or by feed directive in poultry. If these products are approved in the future, strict compliance with regulations will be done with the same policies set for other antimicrobial use and under the guidelines of AMDUCA (1).
5. Extra label antimicrobial therapy must be prescribed only in accordance with the Animal Medicinal Drug Use Clarification Act amendments to the Food, Drug, and Cosmetic Act and its regulations.(1)

   Veterinarians in integrated poultry companies should strive to use antimicrobials at labeled indications and dosage. When prescribing extra label use of antimicrobials, it must always be performed in compliance with AMDUCA (1).
6. Veterinarians should work with those responsible for the care of animals to use antimicrobials judiciously, regardless of the distribution system through which the antimicrobial was obtained.

   Veterinarians in the poultry industry are responsible for the production of poultry at the breeder and meat bird level involving multiple complexes in different geographic locations. Veterinarians work closely with service technicians, service persons, and production managers and are in close contact with all individuals responsible for the use of therapeutic antimicrobials. These individuals are trained in disease recognition and medication strategies. The veterinarian, however, will always be responsible for the initiation and evaluation of antimicrobial therapy.
7. Regimens for therapeutic antimicrobial use should be optimized using current pharmacological information and principles.

   Continuing education programs by the American Veterinary Medical Association, the American Association of Avian Pathologists, and technical updates from pharmaceutical technical service veterinarians keep poultry veterinarians and managers up to date on current information regarding antimicrobial use.
8. Antimicrobials considered important in treating refractory infections in human or veterinary medicine should be used

in animals only after careful review and reasonable justification. Consider using other antimicrobials for initial therapy.

Poultry veterinarians recognize the importance of antimicrobial resistance in both human and veterinary medicine. Important antimicrobials used in both poultry and humans are held in reserve to minimize the rate of resistance development.

9. Use narrow spectrum antimicrobials whenever appropriate.

   Narrow spectrum, bactericidal antimicrobials are chosen when culture and sensitivity results suggest therapeutic success.

10. Use culture and susceptibility results to aid in the selection of antimicrobials when clinically relevant.

    Before antimicrobial therapy is initiated, based on mortality and morbidity, typically affected birds are euthanized and samples taken for bacterial culture and sensitivity. This is common practice in the poultry industry today. The poultry veterinarian uses this information to make informed decisions regarding the appropriate antimicrobial therapy to be initiated. This information is kept as part of the flock and farm history to determine changes in antimicrobial susceptibility patterns on farms.

11. Therapeutic antimicrobial use should be confined to appropriate clinical indications. Inappropriate uses such as for uncomplicated viral infections should be avoided.

    Viral, fungal, and other nonbacterial infections are not treated in poultry with antimicrobials. Veterinarians pay special attention to disease outbreaks to determine whether and when antimicrobial therapy is warranted. Every effort is made to address disease outbreaks with other disease management strategies prior to the initiation of antimicrobial therapy. Mortality and morbidity are monitored closely; diagnostic evaluations are performed to confirm bacterial involvement prior to antimicrobial therapy.

12. Therapeutic exposure to antimicrobials should be minimized by treating only for as long as needed for the desired clinical response.

    Due to the cost of antimicrobial use in poultry, veterinarians and service technicians closely monitor antimicrobial treatments to minimize antimicrobial therapeutic exposure in flocks. Flocks are treated for the desired clinical response avoiding prolonged use of antimicrobials. Morbidity and mortality are used to base clinical judgments as to duration of therapy.

13. Limit therapeutic antimicrobial treatment to ill or at-risk animals, treating the fewest animals indicated.

    In population medicine involving flocks in a disease outbreak, all birds are not infected at the same time with the disease to which antimicrobial therapy is warranted. However, birds in the same house are at risk to the same primary disease. Only birds within the same house, ill, or at risk are treated. Adjacent houses, not clinically affected with disease, are not treated. Cost figures for medication usually are maintained to the one-hundredth of a cent per pound of live weight. If therapeutic antimicrobial intervention is not cost effective, and a low number of birds are infected per house, the cost of treatment usually will dictate that no antibiotics be used at all.

14. Minimize environmental contamination with antimicrobials whenever possible.

    Every effort is made to avoid environmental contamination with antimicrobials. The cost of antibiotics generally ensures that the antimicrobial is used specifically in the diseased flock and not introduced into the environment unnecessarily.

15. Accurate records of treatment and outcome should be used to evaluate therapeutic regimens.

    Record keeping is an integral part of the integrated poultry industry. Production records including medication costs, evaluation, and outcome are kept and placed in the history of the farm for future reference in determining any changing antimicrobial susceptibility patterns.

Summary

Judicious antibiotic therapy includes proper diagnosis, knowledge of antibiotic properties, dosage, spectrum, interactions, and early initiation of treatment. It is not as simple as offering the drug to a poultry flock. The limited arsenal of drugs available for poultry makes it imperative that we combine an accurate diagnosis with antibiotic knowledge to result in the most cost effective approach to disease treatment.

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Chapter 2

Host Factors for Disease Resistance

Introduction

J. M. Sharma

Domesticated and wild birds are vulnerable to many microorganisms that contaminate the environment in which they live. The microorganisms include viruses, bacteria, fungi, and parasites. In confined houses, such as the ones used for intensive rearing of commercial poultry, the concentrations of microorganisms can reach very high levels. Often, these microorganisms are pathogenic and invasive and can cause severe clinical disease or death. The birds manage to survive the microbial challenge primarily because their immune systems provide protection against infection and unrestricted replication of microorganisms. Without effective immunity, the life span of birds would indeed be very short, and commercial poultry production would come to a halt. Because of the importance of immunity in health, the study of the mechanisms of immunity has received much attention within the last few decades, and many pioneering observations have been made that have lead to effective disease-control strategies. One of the most important contributions of immunity to human and animal health has been the development of vaccines that have dramatically reduced the incidence of infectious diseases. Although the avian immune system has not been studied as extensively as that of mammals, important advances have been made. In the first subchapter, a broad outline of the basic elements of the avian immune system is presented. Although great similarities exist between immune mechanisms of the birds and mammals, there are also interesting differences. These differences are identified and briefly described.

In the second subchapter, the role of genetics in regulating immune-mediated resistance to disease is covered. Genetic background of a host determines how the immune response to a given microorganism will evolve, and, ultimately, whether protective immunity will be generated. This is well demonstrated by the great variation that is often observed among individuals within a population in their response to a common disease agent. Some individuals may succumb to infection and die, whereas others may show no phenotypic signs of infection. This wide variation in response to the same agent is attributed to an intrinsic polymorphism of genes that regulate the expression and interaction of various components of the immune system.

Although genetic resistance to a disease is a multigenic trait, in commercial chicken populations, resistance or susceptibility to disease is often attributed to the genes that regulate the major histocompatibility complex (MHC). The MHC encodes a set of cell surface proteins that are necessary for antigen recognition by T cells and, consequently, the capability of T cells to generate specific immunity. The MHC proteins are genetically diverse and polymorphic. Association of specific MHC haplotypes with disease resistance has been exploited by designing breeding programs that select for resistance. Recently, the entire chicken genome has been sequenced, which has provided new opportunities to identify and manipulate genes that control immunity and resistance to disease. The second subchapter provides information on how recent advances in molecular technology have facilitated the study of genetic linkages to disease resistance. This new information is likely to have a significant impact on commercial poultry production.

Avian Immune System

J. M. Sharma

Introduction

The immune system plays a critical role in defending birds against environmental pathogens. The overall organization and mechanisms of immunity in birds are quite similar to those in mammals. Early studies on the bursa and the thymus of chickens provided some of the basic information that led to the identification of the dichotomy of the immune system into B and T cell compartments. The recognition of this dichotomy initiated an era of extensive research on the mechanisms of immunity in mammalian, avian, and amphibian species. This influx of research activity that began about five decades ago continues unabated and is responsible for making immunology one of the fastest

growing branches of biology. Emerging concepts of immune mechanisms are constantly being revised by new information.

The immune system of birds, as of mammals, is complex and includes a number of cells and soluble factors that must work in concert to produce a protective immune response. A properly functioning immune system is of special importance to birds because commercial poultry flocks are raised under intensive rearing conditions. Under such conditions, the flocks are vulnerable to the rapid spread of infectious agents and disease outbreaks. A variety of vaccines must be used, often repeatedly, to protect the flocks against environmental exposure to virulent organisms. The protective efficacy of a vaccine is dependent upon a vigorous immune response against the organism(s) present in the vaccine. If animals are immunosuppressed and respond poorly to a vaccine, the flock health is placed in jeopardy. The understanding of how the immune response is generated is of interest, as is the knowledge of how to protect flocks from stresses that may induce immunosuppression.

This subchapter is not a comprehensive review and is intended to provide a broad overview of selected aspects of the avian immune system. For more detailed information, the reader should consult several books and reviews (17, 18, 28, 35, 81, 85, 87, 101).

Anatomy of the Immune System

The immune cells reside in primary lymphoid organs (PLO) or secondary lymphoid organs (SLO). The thymus and the bursa of Fabricius, respectively, are the PLO where T and B cell precursors differentiate and undergo maturation. The thymus is an elongated, multilobular structure located along the length of both sides of the trachea with some lobes extending into the anterior thoracic cavity (Fig. 2.1A). Thymic lobes are divided into lobules; each lobule has a peripheral cortical area in which lymphocytes are densely packed and a central medullary area in which the lymphocytes are less densely packed (Fig. 2.1B). Bursa of Fabricius is a sac-like extension of the hindgut and is located above the cloaca (Fig. 2.1C). Bursa of Fabricius is organized into follicles, each follicle is filled with lymphocytes. As in the thymus, the lymphocytes are arranged into a peripheral cortex and a central medulla (Fig. 2.1D).

Functional immune cells leave the PLO and populate SLO, the principal sites of antigen-induced immune response. SLO, characterized by aggregates of lymphocytes and antigen-presenting cells, are scattered through the body (Fig. 2.2). Examples of SLO include spleen, bone marrow, gland of Harder (located ventral and posteriomedial to the eyeball), conjunctival-associated (CALT), bronchial-associated (BALT), and gut-associated lymphoid tissue (GALT). Bursa may also serve as SLO. Chickens lack the mammalian equivalent of lymph nodes but have lymphoid nodules along the course of lymphatics.

General Features of the Avian Immune System

An outline of the mechanisms birds use to defend against pathogens is presented in Figure 2.3. Birds have well-developed innate defense mechanisms. Physical barriers such as skin or normal mucosal microflora prevent pathogens from entering the body. For the pathogens that enter the body, the first line of defense is provided by innate immune mechanisms such as phagocytic cells that include heterophils and macrophages (71), complement (47), and natural killer (NK) cells (25, 89).

Innate Immunity

Macrophages that are scattered extensively in the tissues of the host are the first line of defense against pathogens that cross the physical barriers. These cells phagocytize and destroy the invading pathogens and prevent active infection. Macrophages and dendritic cells, another phagocytic cell present in the tissues, respond to the encounter with the pathogen by secreting cytokines and chemokines that initiate a local inflammatory process. Heterophils and serum proteins accumulate at the site of inflammation in an attempt to localize and destroy the pathogen. The recognition of pathogens by the cells of the innate immune system, such as macrophages, neutrophils, and dendritic cells, is facilitated by the presence of toll-like receptors (TLRs) on their surface (23, 33, 54). TLRs are membrane proteins that recognize common antigenic patterns expressed by many pathogens. The interactions of TLRs on macrophages and dendritic cells induce these cells to produce cytokines and chemokines.

Complement is a heat-labile component present in the normal plasma of birds. The complement system is an important and essential part of defense against bacterial pathogens. The activation of the complement system results in the production of a series of proteins. Some of these proteins bind covalently to bacteria. This binding results in bacterial death or enhanced bacterial phagocytosis and destruction. The complement system can be activated by a number of different pathways. The most commonly known are the classical complement pathway (CCP) and the alternate complement pathway (ACP). In the CCP, the complement system is activated by antibodies bound to the surface of the pathogen. In the ACP, a spontaneously activated complement protein destroys bacteria by attaching to bacterial surface. The knowledge of the avian complement system is far behind that of the mammalian complement system. Some of the important biological and molecular characteristics of the avian complement are listed in Tables 2.1 and 2.2.

NK cells are non-T, non-B lymphoid cells that are cytotoxic for virus-infected and tumor cells. These cells are present in normal animals and do not need to be induced by immunization. Avian NK cells express surface CD8αα homodimer and are large granular lymphocytes, morphologically similar to their mammalian counterparts. The NK cytotoxic activity is not restricted by the MHC. In chickens, intestinal epithelial cells are particularly rich in NK cells, although these cells are also present in spleen and peripheral circulation (25). A monoclonal antibody that reacts specifically with intestinal NK cells has been described (25). Studies on the distribution of NK cells suggest that precursors of these cells originate in the bone marrow and migrate to spleen and intestinal epithelium where they acquire functional maturation. The *in vivo* NK cell expression in chickens varies with the age, genetic background, exposure to infectious agents, or presence of tumors (89).

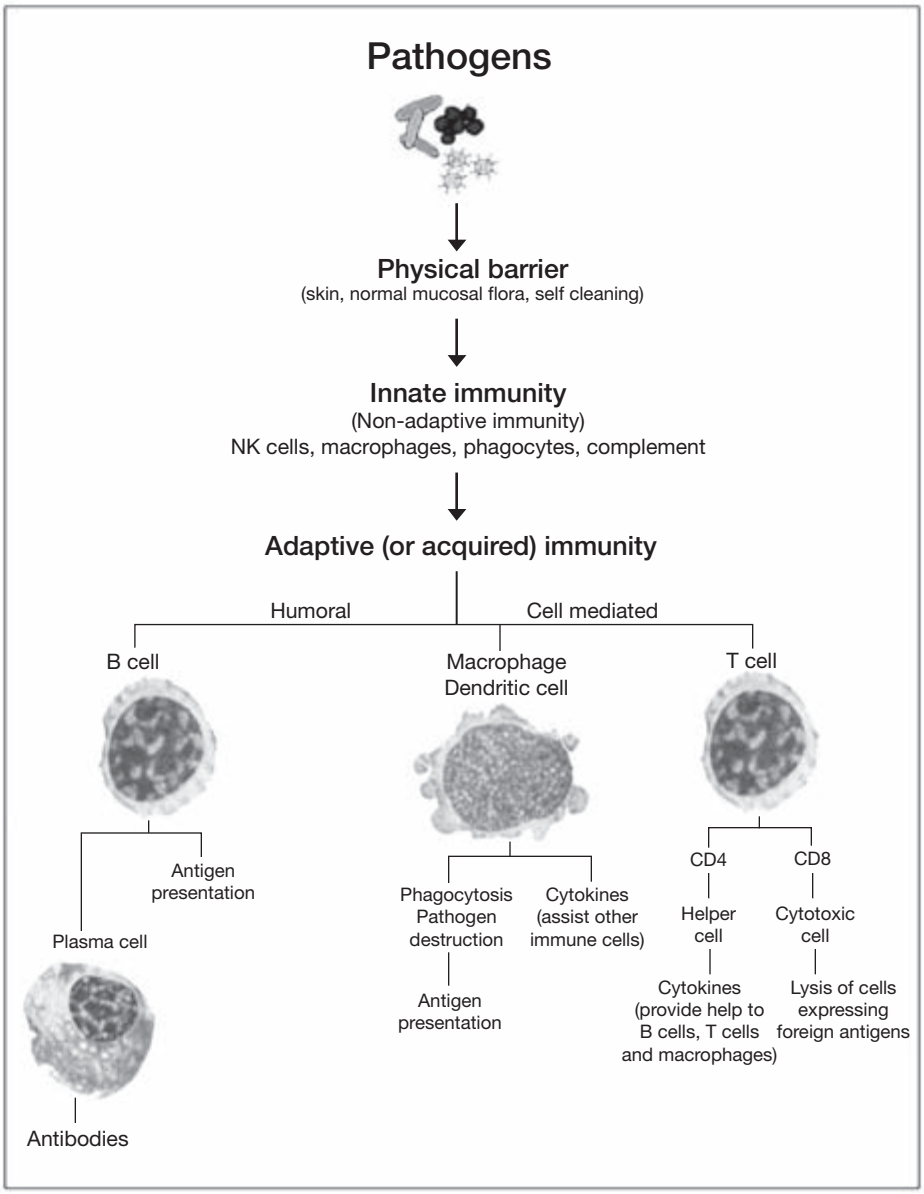


Fig. 2.3. Physical and immunological mechanisms of defense against pathogens in birds.

NK cells and certain other effector cells may also induce target cell lysis if the target cells are coated with antibody. The antibody molecules present on the surface of target cells interact with Fc receptors present on NK cells, and this interaction triggers the cytotoxic attack against the target. The destruction of an antibody-coated target cell is called *antibody-dependent cellular cytotoxicity* (ADCC). The ADCC activity contributes to host defense and has been detected in several avian species (90).

Pathogens that cannot be denied entry by physical barriers or controlled by innate immune defense mechanisms initiate a specific immune response (adaptive immunity). *Adaptive immunity* is highly specific to the agent that stimulates its development whereas nonadaptive or innate immunity is nonspecific. Cells mediating specific immunity retain "memory" of their encounter with the pathogen even after the pathogen has been cleared from the body and detectable immune response has subsided. Memory cells respond to the subsequent exposure to the same pathogen by initiating a rapid and highly effective immune response. Booster vaccinations, used routinely in poultry, take advantage of this memory response.

Adaptive immunity is mediated by a variety of cells, the most important of which are T cells, B cells, and macrophages. T cells, the principal cells of cell-mediated immunity (CMI), recognize foreign antigens after the antigens (such as microorganisms) have been processed by antigen-presenting cells (APC). Macrophages, dendritic cells, and B cells are among the most important APC. The APC generally break down complex antigens and present to T cells small fragments of the antigen in conjunction with the

Table 2.1 Molecular properties of avian complement components.

Component	Species	Whole molecule (kDa)	Separate chains (kDa)	Approximate serum concentration (μg/ml)	Reference
C3	Chicken	185	118	...	(50)
...	...	...	68	...	...
...	...	180	116	500	(61)
...	...	...	67	...	...
...	Quail	183	110	...	(36)
...	...	...	73	...	...
Factor B	Chicken	95	...	50–100[a]	(46)
Clq	Chicken	504	6 H 25.9	50–70	(108)
...	...	...	6 H 24.8	...	...
...	...	...	6 H 24.8	...	...

From Koppenheffer, T.L. 1998. Complement. In J.M. Sharma (ed.). Avian immunology. In P. P. Partoret, P. Griebel, H. Bazin, and A. Govaerts (eds.). *Handbook of Veterinary Immunology*. Academic Press. With permission.
[a]Estimated from data in Koch (46).

Table 2.2 Characteristics of avian complement.

- Antibody-independent ACP activity is demonstrable *in vitro.*
- Microbial parasites activate the ACP *in vivo.*
- The ACP is activated by avian antibodies.
- Hemagglutinating levels of Ab produce maximum lysis.
- CCP activity is difficult to demonstrate.
- Both the ACP and CCP might utilize factor B.
- Cobra venom factor does not uniformly activate avian C.
- The level of hemolytic C in chickens is MHC-linked.

From Koppenheffer, T.L. 1998. Complement. In J.M. Sharma (ed.). Avian immunology. In P. P. Partoret, P. Griebel, H. Bazin, and A. Govaerts (eds.). *Handbook of Veterinary Immunology*. Academic Press. With permission.

MHC molecules. T cells and the APC must share the same MHC before T cells can recognize and react to the antigen being presented.

The MHC molecules are glycoprotein receptors coded by the genes within the MHC. The chicken MHC, also referred to as the B locus, is much smaller than the mammalian MHC and contains only 19 genes in comparison with the human MHC that contains more than 200. The organization of the chicken MHC is also quite different from that of the mammalian MHC (40). The B locus consists of at least 3 loci: BF that encodes class I antigens, BL that encodes class II antigens, and BG that encodes class IV antigens. Class I and class II molecules are highly polymorphic and are critical for antigen presentation by the APC. The BF molecules (class I antigens) are present on a wide variety of nucleated cells including erythrocytes. The expression of the BL molecules (class II antigens) is much more restricted. These molecules are expressed on macrophages, dendritic cells, monocytes, B cells, and activated T cells.

Whereas T cells require that the antigen be processed before it can be recognized, the recognition of an antigen by B cells is not dependent on prior processing. B cells can recognize the antigen as it interacts with immunoglubulins that project from the cell surface. B cells are responsible for humoral immunity and produce antibodies against the antigen.

Most microorganisms stimulate both CMI and humoral immunity although the type of immunity most critical for defense may vary with the microorganism. Some of the important features of the CMI and humoral immunity are discussed in the following section.

Adaptive Immunity

Cell-mediated Immunity (CMI)

T cells are the most important cells of CMI. Many subpopulations of T cells with diverse functions have been identified in chickens. These subpopulations express unique surface antigens that can be detected with monoclonal antibodies. Table 2.3 shows a list of monoclonal antibodies that can recognize some of the surface markers of chicken T cells. This table will undoubtedly undergo periodic revisions as new antibodies are developed.

As in mammals, avian T cells have two surface receptors that bind antigens: T cell receptor (TCR)αβ or TCRγδ. Chickens have a higher proportion of γδ T cells than mice or humans and may reach 30–50% of circulating lymphocytes (93). Both types of TCRs (αβ and γδ) are closely associated with another molecule called CD3, which is present on all T cells. Only the TCR portion of the TCR-CD3 complex interacts with the antigen. The CD3 molecule, which is composed of a complex set of proteins, transmits to the cell the signal of antigen/TCR interaction. The TCR molecules are diversified by rearrangement of single V, D, and J segments derived from multiple polymorphic copies of genes. The chicken TCRαβ locus is different from that of mammals and contains two Vβ families: $V_{\beta 1}$ and $V_{\beta 2}$ (9).

Surface molecules CD4 and CD8 differentiate two important functional subsets of T cells. CD4 is expressed on the surface of helper T (T_H) lymphocytes, whereas CD8 is expressed on the sur-

Table 2.3 Monoclonal antibodies for chicken T lymphocyte antigens.

Antigen	Monoclonal antibodies	Molecular mass (kDa)	Homology (%)	Distribution	References
ChT1	CT1, CT1a, $T_{10}A_6$, RR5-89, Mul83	63 and 45 and dimers	0	Thymocytes, some T lymphocytes	(5, 8, 30)
CD3	CT3	20,19,17,16	36–40	All T lymphocytes	(4, 11)
CD4	CT4, 2–6, 2–35	64	23	Subpopulation of αβ T lymphocytes and thymocytes	(6, 58)
CD5	2–191, 3–58	64	38	T and B lymphocytes	R. Koskinen and O. Vainio, unpublished data
CDw6	S3	110	...	Splenic γδ and most αβ T lymphocytes, some thymocytes	...
ChT6	INN-CH-16	50	...	Activated T lymphocytes	(78)
ChT7	...	110	...	Activated T lymphocytes	(52)
CD8α	CT8, EP72, 11–39, 3-298, AV12, AV13, AV14, CVI-ChT-74.1	34	37	Subpopulations of α, β, γδ and NK-like T lymphocytes and thymocytes	(6, 58, 64, 102)
CD8β	EP42	34	34	Subpopulation of α, β T lymphocytes	...
ChT11	A19	120, 90, 28	...	Intestinal and activated splenic T lymphocytes	(27)
CD28	2–4, 2–102, AV7	40	50	αβ T lymphocytes	(103, 109)
γδ TCR	TCR1	50, 40	30-33	γδ T lymphocytes	(93)
$\alpha\beta_1$ TCR	TCR2	50, 40	26-35	Subpopulation of αβ T lymphocytes	(12, 14)
$\alpha\beta_2$ TCR	TCR3	48, 40	26-35	Subpopulation of αβ T lymphocytes	(7, 10)

From Jeurissen, S. H. M., O. Vainio, and M. J. H. Ratcliffe. 1998. Leukocyte markers in the chicken. In J. M. Sharma (section ed.). Avain immunology. In P. P. Partoret, P. Griebel, H. Bazin, and A. Govaerts (eds.). *Handbook of Veterinary Immunology*. Academic Press. With permission.

face of cytotoxic T lymphocytes (CTL). Great interspecies variation exists in the relative proportions of circulating CD4 and CD8 cells.

Helper T (T_H) Cells

T_H cells (CD4+ cells) recognize processed exogenous antigens in conjunction with MHC II and other costimulatory molecules. When the TCR on the surface of T cells comes in contact with the specific antigenic fragment on the surface of the APC, T cells become activated and proliferate and initiate an immune response directed against the antigen.

Studies in mammals have shown that antigen-induced activation stimulates T_H cells to differentiate into two types of effector populations: T_H1 and T_H2. Differentiation of T_H cells into T_H1 or T_H2 populations is determined by the nature of the stimulating antigen and is mediated by soluble proteins called *cytokines* (see below). Intracellular antigens such as those accumulating within macrophages, dendritic, and other cells stimulate the differentiation of T_H1 cells, whereas extracellular antigens stimulate the differentiation of T_H2 cells. T_H1 effector cells promote proliferation of CD8+ CTL, activate macrophages, enhance their microbicidal activity, and facilitate B cells to produce antigen-specific antibodies with strong opsonizing properties. The principal function of T_H2 effector cells is to help B cells to produce antigen-specific immunoglobulins of various isotypes. Although definitive data are lacking, strong indications show that a similar dichotomy of activated T_H effector cells into T_H1 and T_H2 may also occur in the chicken (19).

Cytotoxic T Cells (CTL)

Most CTL express CD8 surface molecules. A small proportion of mammalian CD4 T cells may also have cytotoxic activity, although the presence of avian CD4 T cells with cytotoxic ability has not been documented. CD8+ CTL recognize endogenous antigens in conjunction with MHC I (59). Internalized antigens such as viruses are degraded into small peptides by a large proteolytic complex called a proteasome. Small antigen peptides, usually 7–13 amino acids long, are then transported to the endoplasmic reticulum where the peptides become attached to MHC I. The peptide-MHC I complex is then transported to the cell surface for possible recognition by antigen-specific CTL.

One of the most important functions of CTL is the elimination of virus-infected cells. Because most nucleated cells express surface MHC I, virus infection of almost any cell can lead to potential recognition and lysis by CTL. *In vitro* assays to quantitate

CTL in chickens have been difficult to establish because of MHC restriction of effector and target cells. Despite this difficulty, CTL activity has been shown to regulate pathogenesis of avian viral and neoplastic diseases (15, 35, 79).

Avian Cytokines

Cytokines are small, biologically active proteins secreted by a number of cells, most notably immune cells. Cytokines bind to specific receptors on the surface of target cells and regulate immune response by signaling between cells. Receptor-bound cytokines and other membrane-associated molecules often act together to stimulate the effector function in a target cell. T cells, B cells, macrophages, and dendritic cells all secrete cytokines. Cytokines produced by T_H cells in particular play a key role in modulating an immune response. T_H1 cells, which promote a CMI response, produce predominantly IFN-γ, which activates macrophages and enhances destruction of cell-associated pathogens. Other major cytokines produced by T_H1 cells include IL-2 and tumor necrosis factor-α (TNF-α). IL-2 is critical for the proliferation of a number of immune cells including T_H, T_H1, and T_H2 cells, CTL, NK cells, and B cells. The T_H1 cell activity and cytokine secretion is stimulated by IL-12 and IL-18, both produced by macrophages, dendritic cells, and B cells.

Cytokines produced by T_H2 cells, which promote B cell activation and antibody production, include IL-4, IL-5, and IL-10. IL-1, a product of a number of cell types, most notably macrophages, stimulates the T_H2 cell activity.

Within the last few years, a number of avian cytokines have been isolated and characterized. Genes coding for avian cytokines and their receptors have been cloned and sequenced (Table 2.4). The biological activity of avian cytokines is generally quite similar to their mammalian counterparts, although avian cytokines show little cross-species biological reactivity.

Humoral Immunity

Immunoglobulins (Ig) or antibodies secreted by B cells constitute the principal component of humoral immunity. Antibodies may be present in many body fluids but are most readily detected in the serum or the plasma fraction of blood. Exposure of birds to microorganisms stimulates the production of specific antibodies, which, in turn, react with microorganisms and hasten their destruction. The three mechanisms by which antibodies contribute to defense against pathogens are as follows: *Neutralization*, in which antibodies bind to and neutralize specific pathogens, particularly viruses. Neutralized viruses are unable to attach to surface receptors of target cells and are thus prevented from replication. *Opsonization* which includes bacterial pathogens that can replicate extracellularly, and are more readily internalized and destroyed by phagocytes if the pathogens are coated with antibodies. *Complement activation,* in which antibodies bound to the surface of pathogens, can activate complement and produce new complement proteins. The complement proteins attach to receptors on phagocytes, which facilitate the phagocytosis and destruction of pathogens.

Chickens have three main classes of immunoglobulins: IgM, IgG, and IgA (Table 2.5). Figure 2.4 shows the typical structure of an Ig molecule. All Ig molecules have two distinct types of polypeptide chains. The smaller polypeptide chain, called the "light chain," is common to all classes of Ig, whereas the larger chain, called the "heavy chain," is structurally distinct for each class or subclass of Ig. Covalent and noncovalent forces connect the two chains. The structure of the heavy chain determines the biological function of each class of Ig. Genes encoding all three classes of avian Ig have been cloned and sequenced, which has facilitated the generation of recombinant avian and chimeric antibodies *in vitro* and the expression of recombinant avian Ig in plants (16, 26, 60, 68, 77, 106).

IgM is found on the surface of most B cells and is the first antibody produced following primary immunization. As the immune response progresses, the IgM-producing cells stop IgM production and start the production of IgG or IgA. This phenomenon is called "class switch." The antigen-binding ability of the antibodies does not alter during or after the switch. The "class switch" occurs because the antibody-producing B cell begins to splice the variable (V) region genes (V genes) to the constant (C) region genes (C genes) of the heavy chain of a different class of Ig. Cytokines including IL-4, TGF-β and IFN-γ stimulate the B cell to undergo class switch (21).

A typical immune response of a chicken begins with IgM production. After some time, IgM production switches over to IgG production. IgG is also the principal antibody produced after secondary immunization and is the predominant Ig class in chicken blood. Because avian (and also amphibian, reptile, and piscine) IgG is larger than its mammalian counterpart, the chicken IgG is often called IgY (104). Figure 2.4 compares the relative structure of mammalian and avian IgG. Molecular cloning data suggest that IgY may be the ancestral precursor of mammalian IgG and IgE (104).

IgA is the most important Ig involved in mucosal immunity. Chicken secretory IgA (sIgA) exists as a dimer in mucosal secretions, whereas circulating IgA is polymeric or monomeric. IgA complexes with a secretory component present on the surface of mucosal epithelial cells to form sIgA (107). The acquisition of the secretory component protects IgA from proteolytic digestion in the gut. IgA is most concentrated on mucosal surfaces, although small quantities may be found in the circulation. Bile is also a rich reservoir of IgA in birds. IgA protects mucosal surfaces against pathogens, particularly viruses, by neutralizing and preventing their attachment to receptors on target cells.

As noted earlier, B cells use surface Ig to bind to antigens. Each B cell produces only one type of heavy and light chain and is committed to one kind of antigenic determinant. Thus, for an antigen to initiate antibody production and clonal expansion, the antigen must interact with a B cell that expresses the homologous Ig receptor. Potentially thousands of antigens and millions of antigenic shapes are in the environment. How does the immune system maintain an inventory of B cells with such a wide variety of antigenic specificities? This is accomplished by a number of genetic mechanisms during the development and maturation of B cells. In mammals, Ig gene rearrangement leads to extensive Ig diversity. In the chicken, because of a relatively small numbers of Ig genes, the rearranged genes must undergo a process called *gene conversion* to attain needed diversity (21). In gene conver-

Table 2.4 Avian cytokine genes identified.

Cytokine gene cloned	Avian species	Reference
IFN-α	chicken, turkey, duck	(83, 84, 96)
IFN-β	chicken	(92)
IFN-γ	chicken, turkey, Japanese quail, guinea fowl, duck	(22, 31, 38, 57, 82)
IL-1β	chicken	(105)
IL-2	chicken, turkey, Japanese quail, duck, goose	(37, 51, 55, 94, 95, 111)
IL-3	chicken	(2, 37)
IL-4	chicken	(2, 37)
IL-5	chicken	(37)
IL-6	chicken	(37, 100)
IL-7	chicken	(37)
IL-9	chicken	(37)
IL-10	chicken	(37, 75)
IL-12	chicken	(3, 20, 37, 100)
IL-13	chicken	(2, 37)
IL-15	chicken	(13, 37, 55)
IL-16	chicken	(37, 63)
IL-17	chicken	(63)
IL-17A ,B,D.F	chicken	(37)
IL-18	chicken, turkey	(37, 80)
IL-19	chicken	(37)
IL-21	chicken	(37)
IL-22	chicken	(37)
IL-26	chicken	(37)
IFN-λ1 (IL-29),- λ 2 (IL-28A), - λ 3 (IL-28B)	chicken	(37)
Granulocyte colony-stimulating factor (CSF3)	chicken	(2, 37)
GM-CSF	chicken	(37, 76)
Stem cell factor	chicken	(110)
Chicken MGF	chicken	(53)
TGFβ1	chicken	(34)
TGF-β2,TGF-β3	chicken	(37)
TGFβ4	chicken	(67)
Lymphotactin	chicken	(100)
MIP-1β	chicken	(70)
Chemokines (K60, K203)	chicken	(91)
Chemokines	chicken	(32)
chXCL1,chCCLi5,chCCLi6	chicken	(37)
chCCLi7,chCCLi8,chCCLi9		
chCCLi10,ccCCLi1,ccCCLi2		
ccCCLi3,chCCLi4,chCCL17		
chCCL19,chCCL20,chCCL21		
chCXCLi1,chCXCLi2 (IL-8)	chicken	(72)
chCXCLi3,chCXCL12,	chicken	(97)
chCXCL13L1,chCXCL13L2	chicken	(29)
chCXCL13L3,chCLCXL14		
chCX3CL1	chicken	(1, 37)
Stromal cell derived factor-1	chicken	(1)
ChTL1A	chicken	(1)
LPS-induced TNF-alpha factor (LITAF)	chicken	(37)
TRAIL	chicken	(1, 37)
TNFRII	chicken	(37)
TRAF5		
VEGF		
CD30L		
CD40L		
BAFF		

(Data for this table provided by Mahesh Khatri, University of Minnesota, St. Paul, MN 55127.)

Table 2.5 Properties of chicken immunoglobulin isotypes.

Isotype	*Heavy chain (kDa)*	*#H chain Ig domains*	*Homology to mammalian*	*Serum concentration*	*Sources*	*Structure and comments*
IgM	70 kDa	5	About 30%	1–2 mg/ml	Serum	900 kDa, consistent with heavily glycosylated $(\mu_2L_2)_5$ plus a J chain
			78% for TM*		Cell surface	μ_2L_2 monomer of membrane IgM, no J chain
IgG	67 kDa	4	30–35%	5–10mg/ ml	Serum	175 kDa, γ_2L_2 monomeric form
					Egg Yolk	γ_2L_2, high concentrations (10 mg/ml) of IgG are found in egg yolk(low concentrations in egg white)
IgA	65 kDa	4	32–41%	≅3 mg/ ml	Serum	170 kDa, α_2L_2-monomeric form without J-chain
					Bile	350 kDa, consistent with $(\alpha_2L_2)_2$ plus a J-chain
					Mucosa (tears, saliva)	600–700 kDa, consistent with $(\alpha_2L_2)_4$ plus a J-chain

*TM refers to the transmembrane and cytoplasmic domains of chicken sIgM.
(From Demaries, S.L. and M.J.H. Ratcliffe. Cell surface and secreted immunoglubulins in B cell development. In: J.M. Sharma, ed., Avian Immunology. In: Partoret P.P., P. Griebel, H. Bazin, and A. Govaerts, eds., *Handbook of Veterinary Immunology*, Academic Press, 1998. With Permission).

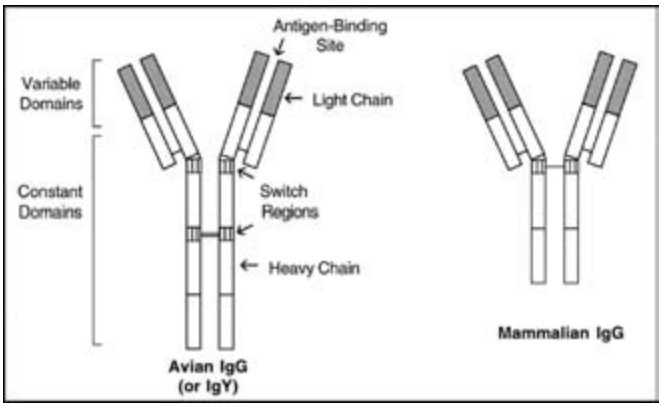


Fig. 2.4. Typical structure of an Ig molecule and comparison between avian and mammalian IgG molecule.

sion, the rearranged light and heavy chain gene complexes acquire clusters of chromosomal pseudogenes. Large segments of highly homologous pseudogenes are present in the vicinity of light and heavy chain genes in the chicken chromosome (73, 74).

Maternal Transfer of Immunity

Transmission of immunity from the hen to the newly hatched chick is critical for protecting the chick against infections during early life. In chickens, Ig are the principal mode of transfer of immunity. There is little evidence that the mother's immune cells are passed on to the embryo. Ig from hen's circulation are deposited in the superficial epithelial and glandular cells of the oviduct (45). From the oviduct, IgG is transferred into the maturing oocyst in the ovarian follicle and accumulates in the yolk sac. Ig produced locally in the oviduct likely constitutes an insignificant proportion of the transferred Ig. The developing chick acquires maternal IgG from the yolk sac. IgA and IgM are transferred via the amniotic fluid. The developing embryo swallows IgA- and IgM-containing amniotic fluid.

Assays to Measure Immunity

NK Cells

NK cell assays are based on *in vitro* cytotoxicity against susceptible target cells (24, 49, 89). The most commonly used targets are the cells of the line LSCC-RP9 (88). These cells were derived from a retrovirus-induced tumor in a B^2B^{15} male chicken (65). The target cells are labeled with ^{51}Cr and incubated *in vitro* with varying concentrations of cell suspension being tested for NK cell activity (effector cells). Two controls are important: a) adding "neutral" cells such as thymocytes to target cells at the same effector:target ratios as used for the effector cells, and b) the use of NK-resistant target cells. After 4 hours of incubation at 37°C, the radioactivity released into the medium is quantitated. Specific cytotoxicity, a measure of NK cell lysis, is calculated by the following formula:

% cytotoxicity = counts per minute (cpm) in target cells mixed with effector cells – cpm in target cells mixed with normal thymus cells / total cpm incorporated in target cells – cpm in target cells mixed with normal thymus cells × 100.

Macrophages

Macrophages, a phenotypically diverse population of cells, are present in almost all tissues. Because most macrophages adhere to substrates, they can be readily isolated from short-term *in vitro* cultures of peripheral blood cells (PBL) or single cell suspensions of spleen (42). Peritoneal macrophages may also be induced in birds by intraperitoneal injections of inflammatory

stimulants such as Sephadex beads. Some of the assays used to assess macrophage functions include a) phagocytosis, b) cytokine production upon stimulation with mitogens (lipopolysaccharide), c) ability to lyse tumor cells, and d) production of nitric oxide (NO) upon activation by T cell-produced cytokines, most notably IFN-γ. Some of the functional characteristics of avian macrophages have been described (41, 42, 62, 66, 71, 86).

T Cells

Most T_H cell assays are based on *in vitro* stimulation of cells with mitogens or specific antigens (43, 44, 99). Stimulated cells proliferate and secrete cytokines. Mitogen-induced proliferation is a common assay of T-cell competence. Concanavalin A (Con A) and phytohemagglutinin (PHA) are the mitogens of choice. These mitogens bind to cell surface glycoproteins on T cells and stimulate the cells to proliferate. In the typical assays, spleen cells, PBL, or diluted whole blood are cultured *in vitro* in medium containing Con A or PHA. After 40 hours of incubation at 37–41°C, the cells are pulse labeled with radioactive thymidine. The incorporation of the label in cellular DNA is quantitated. Actively proliferating cultures incorporate higher levels of radioactivity than non-proliferating cultures. If the proliferative response of the test group of chickens is lower than the response in age-matched healthy controls, the test group is viewed as being deficient in functional T cells. This general conclusion should be viewed with caution because the mitogen-induced proliferation is not antigen-specific, and response to mitogen is an *in vitro* function of T cells. The *in vivo* relevance of this function to other *in vitro* or *in vivo* functions of T cells is not known, and functional T cells may be prevented from proliferating by non-T suppressor cells or suppressor products present in the culture (43, 69).

T cells recovered from immunized animals may proliferate *in vitro* when co-cultured with the antigen used for immunization (44, 99). This antigen-specific proliferation has been shown with several avian viruses, although the ideal assay conditions are not well established, and the test is not widely used.

Mitogen- or antigen-induced stimulation of T cells *in vitro* also results in the secretion of cytokines. Quantitation of cytokines in the culture medium gives an indication of the functional capability of T cells. A nitric oxide inducing factor (NOIF) test has been described in which macrophage-stimulating cytokines such as IFN-γ can be quantitated. Macrophage line cells are exposed to the test supernatants, and NO concentration in the supernatants is calculated (39).

CTL activity can be measured *in vitro* by co-culturing effector cells with ^{51}Cr-labeled target cells (59). The protocols are quite similar to those described previously for NK cell cytotoxicity assays. The cytotoxic activity of CTL is MHC I-restricted. Therefore, both effector and target cells must come from the same or a genetically compatible bird. Because of this limitation, CTL assay is difficult to perform in outbred populations of birds and remains a research tool.

Certain *in vivo* assays may also be used to assess T-cell functions. The delayed type hypersensitivity assay measures antigen specific response. In this test, an animal immunized against an antigen is intradermally injected with the same antigen. Swelling at the site of the injection comprises a positive response. Local swelling at the site of an intradermal injection of mitogens such as PHA has also been attributed to a non-specific T cell response.

Antibody Levels

Birds exposed to pathogens develop circulating antibodies that generally persist for several weeks after the antigen has been cleared. Detection of these antibodies is much more convenient than detecting cellular immunity, and a number of serologic assays are available to quantitate antibodies. Some of the commonly used serologic tests include agar gel precipitation test, virus neutralization test, immunofluorescence test, hemagglutination inhibition test, and enzyme-linked immunosorbent assay (ELISA). Protocols for conducting these tests have been described (98).

ELISA is by far the most common serologic assay used under commercial settings. Automated technology allows rapid processing of large numbers of serum samples. Computerized data transmission facilitates flock profiling and provides useful information on environmental exposure to pathogens and response to vaccination. ELISA kits that can be used to detect antibodies against most of the common viral and bacterial pathogens of poultry are available commercially.

The transfer of IgG from the yolk sac to the embryo or the hatchling occurs by absorption into the recipient's circulation. Yolk sac is highly vascularized, and IgG is transferred by receptor-mediated endocytosis across the yolk sac epithelium (56). The transfer of IgG begins during the first week of embryonation but occurs most predominantly during the last three days before hatching (48). The transfer from the yolk continues after hatch. Peak levels of maternal IgG in the circulation of the newly hatched chick reach around 2–3 days of age. Maternally derived antibodies decline linearly in the recipient and become undetectable after 2–5 weeks.

Although maternal antibodies are important for the well being of the newly hatched chick, the antibodies may interfere with active immunization with live vaccines. Neonatal or *in ovo* vaccination is often necessary in flocks being raised in heavily contaminated environment. Besides neutralizing the antigen present in the vaccine, pre-existing antibodies may also interfere with the development of active immunity by providing negative feedback to the immune system.

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Genetics of Disease Resistance

Hans H. Cheng and Susan J. Lamont

Introduction

Genetic resistance is alluring from both the industrial and academic viewpoints. With respect to poultry companies, losses due to diseases induced by infectious pathogens continue to be a significant issue and can be the key factor in determining economic viability. This is because pathogens lead to loss or condemnation of birds; inhibit the immune response, making birds susceptible to other pathogens and diseases; divert critical resources from growth and production; add expenses for vaccination programs; and force changes in husbandry practices, all of which increase the cost of production. Furthermore, certain pathogens may cause a disruption in trade between countries or produce a loss of public confidence in food product safety. Consequently, genetic resistance can be a powerful approach in combination with other management practices to eliminate or manage infectious diseases of agronomic interest, especially as a long-term solution in light of the emergence of new and more virulent pathogens and increasing restrictions on the use of antibiotics.

From the academic side, modern molecular genetics has provided an arsenal of new tools for identifying genes and alleles that confer resistance to disease. Some of the complexity of biology, and in particular the immune response, may finally become fully elucidated. It is reasonable to expect that genetics will identify genes, or at least genomic regions containing these genes (known as QTL or quantitative trait loci) that influence complex traits like disease resistance. It is also expected that information will be forthcoming on how these genes function and interact as well as respond to changing environments to control disease. Ultimately, this information will be transferred to poultry companies to generate elite lines with superior disease resistance or better vaccinal response. On the other hand, it is clear that the field is in its formative years, and our ability to predict and model complex traits is limited. And while advancements in biotechnology will continue, technology cannot speed up the maturation rate, generation intervals, the number of progeny produced per day or per bird, and other biologically-limited traits and resources that are required for experimental studies. Consequently, it is anticipated with the rapidly changing landscape of biological knowledge that long-held assumptions will be shattered, requiring revised models and paradigms. Fortunately, the momentum for continued progress in genomics remains high along with the seemingly unending string of technological advancements.

Besides these interests, studies on genetic resistance and genetics in general are the forerunners of change that will undoubtedly occur in all areas of biology including veterinary medicine and diagnostics. With the advent of molecular genetic maps, the genome sequence, and genomics, "discovery-driven research" emerged as the preeminent method for dissecting and understanding complex traits like disease resistance. Consequently, while genetics has always been a field that used a holistic approach to examine the entire organism, with the ability to measure and record millions of data points at the DNA, RNA, and protein levels quickly and economically, the power of the existing and upcoming technologies has and will continue to shift the field toward large-scale unbiased screens using molecular and computational biology and their integration. This does not mean that scientists and clinicians need to become facile in genomic technologies, rather it indicates that knowledge and information can be more readily transferred to other fields.

In this section, we focus on recent advancements in genetic resistance to disease, namely, molecular and quantitative genetics; for reviews on classical genetics or specific genes for disease resistance, see 16, 27, 37, 55, 56, 75, 96. The targeted audience is animal health professionals and others that may not be familiar with molecular or quantitative genetics. We hope to convey the high level of excitement (as well as limitations) in these areas, which has been brought about with the recent release of the chicken genome sequence and other related technologies. To achieve these goals, sections are presented on (1) a review of genetic concepts necessary for a basic understanding, (2) molecular genetic approaches to identify disease-resistance genes, (3) complementary functional genomic approaches that provide insights on the biochemical mechanisms and pathways of disease resistance, (4) brief summaries of genetic resistance and experimental studies for specific diseases, and (5) thoughts on how this information may be applied in poultry breeding to improve host resistance to infectious disease. The emphasis throughout these sections is on basic concepts as given the short history and dynamics of the field, knowledge, and state-of-the art methods will change rapidly. Finally, some topics of general interest (e.g., the chicken genome assembly, use of molecular genetic tools for veterinary diagnostics) are briefly discussed as general interest or tools that could be transferred to veterinary diagnostics.

Review of Quantitative and Molecular Genetics

Classical or Mendelian genetics trains us to think about biology in mathematical (quantifiable) terms by using discrete phenotypes (measurable traits) that are explained by one or a few genetic loci. Certainly, there are "simple" or qualitative traits as evidenced by the large number of loci and alleles, often with interesting and descriptive names that account for plumage color and patterns—e.g., dominant white, slow feathering, naked neck. However, for the majority of traits (phenotypes), there is natural variation with continuous (or quantitative) rather than discrete (or qualitative) phenotypic classes within a population. This phenotypic variation can arise from the segregation of a single gene with multiple alleles (forms of genetic variants), or numerous genes, combined with modulating effects due to interactions with

the environment. The critical difference is that for a quantitative trait, the phenotypic variation among individuals of a specific genotype, is relatively larger than the average phenotypic difference between genotypes.

Genetics strives to understand how the phenotypes of organisms are influenced by their genotypes (genetic makeup). More specifically with modern molecular genetics, we wish to know the relationship between genetic variation and phenotypic variation. In other words, the goal is to determine how variation within a population can be accounted for at the genetic level, which should facilitate selection of the trait in a breeding program. This does not mean to say that a gene with no discernable allelic variation does not contribute to the trait but rather that there is no variation within this population for genetic improvement. At the simplest level, given a genetic component, we wish to know whether different alleles for a given gene or locus give statistically significant differences in a particular trait. With the advent of molecular genetic maps, we can extend this same question to the entire genome. In short, the goal of genetics is to predict what kinds of offspring are produced by a specific cross. More specifically, the major goal of molecular and quantitative genetics is to identify the genes and alleles that account for the observed phenotypic variation within a population. This result is achieved by mapping the traits genetically and by understanding the mechanistic basis of the genetic contributions to phenotype.

The basic genetics concepts, which are described in the following discussion, have been established for many years and utilized well as evidenced by the great progress made by poultry breeding companies. What has changed is the emergence of molecular genetic maps and high-throughput screening of genotypes, which has had a dramatic and positive impact. It is now possible to identify the underlying genetic basis for complex traits such as disease resistance. It is anticipated that many genes and gene products discussed in the prior subchapter will contribute to genetic resistance to disease.

Phenotypic Distributions

Even within individuals with identical genotypes, there is a range of phenotypes. In other words, while an individual's genotype certainly influences its phenotypes, it does not mean that genotype equals phenotype. This can be seen where field crops with the same genetic makeup are very uniform but upon closer inspection, subtle variations in height or other measurable traits are found. This range is due to the fact that traits are not the outcome of the contributing genes only, but normal biological variation, response to the environment, and other factors. For simple and qualitative traits, the phenotypic distributions of unique genotypes may be very tight and not overlap with others to give discrete classes. This is often not the situation when dealing with complex and quantitative traits where a continuum is found. This also implies that individuals with different genotypes may have the same phenotype.

Disease presents unique issues with analysis of quantitative traits, as the definition often varies according to the pathogen. Disease is the unfavorable outcome of the interactions between the host, pathogen, and environments whereby conditions exist to favor pathogen growth and spread. Disease resistance could mean absolute resistance to infection. For example, chickens that are resistant to certain subgroups of avian leukosis viruses (ALV) lack the corresponding cellular receptor for ALV attachment and entry. Alternatively, disease resistance could mean tolerance—e.g., all birds are considered to harbor intestinal parasites—but the resistant birds do not go on to show debilitating symptoms. In other words, the resistant birds did not pass a certain threshold to be classified as diseased. Regardless of the situation, it is necessary to define what disease means for that pathogen.

Disease can also be considered as simply a binary or two-state trait, as a bird is classified as either resistant or susceptible. It would be advantageous for disease to be broken down into continuously variable, quantifiable, and/or measurable components. Using Marek's disease (MD) as an example, besides asking whether a bird gets tumors or enlarged nerves (i.e., diseased), one could monitor the amount of virus after infection, the days until death, and a whole variety of other measurable MD-associated traits. Besides providing additional statistical power, it has the potential advantage that disease resistance can be ascribed to one or more components. It is in the ability to define and quantify disease traits that veterinarian pathologists and immunologists may significantly help studies on genetic resistance.

Heritability

A prerequisite of genetics and breeding (genetic improvement) is that the trait of interest is heritable. This can be determined somewhat by observing whether related birds are more similar to each other than unrelated ones. This assumes that the environmental conditions are the same for all birds. A more direct test would be to take individuals at the two extreme ends of the phenotypic distributions, mate them to birds of similar phenotype, and then determine whether the measurable differences between the two groups of offspring are maintained, thus, inherited. For example, let's say in a population of broilers grown in the same environment under the same feed regiment that birds vary in the percent abdominal fat. To determine whether there is a genetic basis for this variation in abdominal fat, one would mate sires and dams with high abdominal fat and sires and dams with low abdominal fat to produce two sets of chicks. Following the rearing of these chicks in the same environment, if abdominal fat is heritable, then the phenotypic distribution of abdominal fat should differ between the two groups with the chicks resembling their parents in the percent abdominal fat.

This extent of similarity from parent to offspring, or heritability, can be quantified. Variation in a trait within a population results from variation in the genotypes (genetic effect) and variation due to mainly the environment. The genetic variation can be further broken down into what are called the additive and dominance variance components. Suffice it to say that the additive genetic variance accounts for the average effect of each allele of a gene, while the dominance variance component measures deviation from the predicted average of the two alleles. What is normally reported for the heritability of a trait is the amount of phenotypic variation that can be accounted for by the additive genetic variation and is represented as h^2. Values range from 0 (no heritability) to 1, where the trait measures of the parents

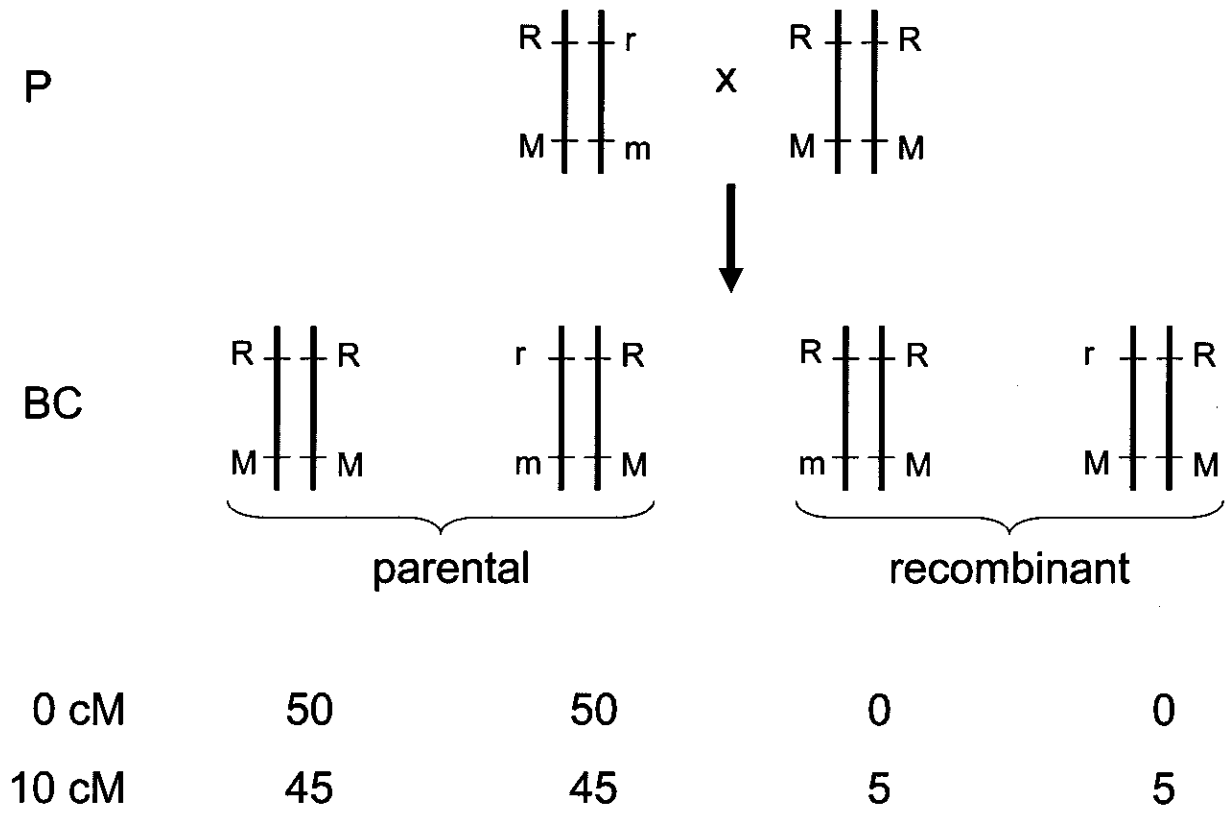


Fig. 2.5. Linkage between a hypothetical virus receptor with defective (R) and functional receptor (r) alleles, and a molecular marker with alleles M and m. In this example, parental (P) chickens that are susceptible (R/r) and resistant (R/R) to the virus are intermated to produce backcross (BC) progeny. If the loci encoding the viral receptor and the molecular marker are completely linked at the genetic level, then the molecular marker will absolutely predict the virus resistance status of the bird as all resistant birds will have the M/M genotype while all susceptible birds will be M/m. However, if loci are linked but can be separated at the genetic level during meiosis, if the distance between the loci is 10 cM, then the molecular marker will again be predicted but only 90% of the time.

would exactly predict the value of the offspring. By knowing the heritability estimate of a trait, breeders can predict the response to selection. Using percent abdominal fat as an example, if the population average was 4%, and we choose parents with 2% abdominal fat, then if the heritability of abdominal fat was 0 (no genetic influence), 0.5 (moderate genetic influence), or 1.0 (absolute genetic influence), the offspring would be expected to have an average of 4%, 3%, and 2% abdominal fat, respectively.

For disease resistance, in general, heritability estimates for specific diseases are reported to be low to moderate (37). However, it is important to note that heritability estimates are for a particular population (set of birds and genetic composition) under a single environment. This may be one of the main reasons why there is a range of reported h^2 for the same traits as the populations and environments are not constant between experiments and estimates. This also shows that heritability is not fixed for any one population and may change following selection (genotypes are altered) or in a different environment.

Linkage

Linkage, or the nonrandom association (co-inheritance) of alleles, is the main weapon in the molecular geneticist's toolbox. It is this ability that can determine whether a disease-resistance gene is nearby or linked to a specific molecular marker. To illustrate this point, let's start with the simplest example of a single gene trait. We'll assume that there is a gene that encodes the cellular receptor to a nasty virus, and virus binding to this receptor is required for cellular infection. The R allele makes a defective receptor, thus, confers resistance to infection and disease while the other allele r makes the normal protein, which confers virus entry and susceptibility. Because the bird needs only one r allele for disease susceptibility, disease resistance is a recessive trait, as a bird would need two R alleles to prevent virus-induced disease. If you mated a susceptible bird with the R/r genotype (chickens are diploid, thus, have 2 alleles for each locus) with a resistant bird (R/R), about half of the progeny should be susceptible with the R/r genotype while the other half should be resistant with the R/R genotype.

To locate the disease-resistance gene, one can test for co-segregation using molecular markers. In Figure 2.5, for 100 backcross (BC) progeny, if we find that all 50 resistant and 50 susceptible birds always have the genotypes of M/M and M/m, respectively, for a marker, then the marker is completely linked with and cannot be separated from the disease-resistance gene. This is not to say that marker M is the disease-resistance gene, but only that the two loci were not separated genetically when the gametes were formed during meiosis.

This also demonstrates that alleles M and R, and alleles m and r, are in linkage disequilibrium (LD). If the marker locus and disease resistance gene were unlinked, then the alleles should randomly segregate. But this is not the case as M occurs more frequently than is expected, and in this case, the marker allele is predictive of the disease resistance gene allele.

But what if the two loci are not absolutely linked? Imagine that of the 50 disease resistant birds, 45 had the M/M genotype and 5 had the M/m genotype. Likewise, of the 50 susceptible birds, 45 had the M/m genotype while 5 had the M/M genotype. Having the M allele is still a very good indicator of disease resistance (or vice versa with the m allele), but it is not 100% accurate. In this case, we can determine the linkage of the two loci by quantifying the percentage of recombinant alleles (non-parental ones—e.g., R and m or r and M). In this example, there are 10 recombinant progeny out of 100, which means that the disease resistance gene and the marker are 10 centiMorgan (cM) away. The genetic term cM reflects the number of recombination events between two loci detected in 100 progeny. In other words, the extent of linkage between these two loci is determined by the amount of genetic recombination that occurs during meiosis, which can be determined by measuring how often particular alleles within each locus are inherited together.

To make the situation even more difficult and to put into perspective the challenge of finding genes for complex disease resistance, imagine now that each disease resistance gene accounts for only a small percent (2–10%) of the total observed variation in the segregating population, as is typical for most diseases. Furthermore, not all R/r birds are susceptible as the r allele may not always be expressed at sufficient levels to allow virus entry. Or not all the birds get challenged with nasty virus as natural exposure is being used. While daunting, these challenges can be overcome with proper and controlled experiments, the use of molecular markers that encompass the entire chicken genome, many progeny to give more statistical power, accurate phenotypes, and biometrical analyses.

Experimental Approaches for Molecular Genetics

The development of genetic maps based on molecular markers defined the birth of genomics. No longer were scientists limited to assaying for genetic effects using markers at undefined locations. Now it was possible to systematically query the entire genome. This ability also changed the way experiments were primarily conducted. Rather than using the reductionist approach of formulating a hypothesis about a specific gene and then testing it, genomics is a discovery-driven field where the end results allow definition of the next series of experiments. The genomics approach is growing in influence and is being reinforced and strengthened with whole genome sequences and high-throughput technologies that deliver quick, accurate, and low-cost data points.

Candidate Genes

In the preceding subchapter, a number of genes and genes products have been identified that modulate the immune response. Many of these immune function genes have or may be good candidates for conferring disease resistance. Consequently, prior knowledge to identify candidate genes for specific disease resistance is a valid method and often the best first choice for testing for a genetic effect. This approach requires a population that is segregating for disease or disease-associated traits, and an informative DNA polymorphism within or close to the gene of interest.

Although a gene and its gene product may be a critical component of the immune response and disease resistance, it is possible that there is no variation or genetic effect attributable to the gene in a specific population. Consequently, the first requirement is to identify polymorphisms (structural variations in the DNA) and alleles of the gene that are segregating within the population. Furthermore, evidence for a genetic effect does not prove causation by that gene but may be a result of linkage between the causal gene and the analyzed gene. Finally, only that specific gene and genomic region is being screened for genetic effects, and no information is generated about other genomic regions that may also influence resistance to the disease.

The MHC represents a unique situation and opportunity. Due to its importance in many diseases, chicken lines have been developed that vary only in the MHC or B locus (1, 5, 40). Thus, these lines have the identical genetic background and carry different and unique MHC haplotypes. So, rather than using a genetic marker to the MHC and asking whether it is associated with disease resistance, these "B congenic" strains can be used to quickly screen for MHC influences on disease resistance by challenging each line with the pathogen of interest and monitoring disease and disease traits. Furthermore, due to the inbred nature of each line, there is less genetic variation that has to be accounted for, which translates into fewer chickens needing to be measured to detect genetic associations with traits.

Whole Genome Scans

With availability of the chicken molecular genetic map, it is possible to screen the entire chicken genome for disease-resistance genes. The general approach is shown in Figure 2.6. In brief, what is called a resource population is identified or produced where birds segregate for disease resistance. Some or all of the animals are measured for disease or disease-associated traits. Likewise, the same animals are genotyped for molecular markers spaced evenly throughout the entire chicken genome. Statistical analyses are performed to see whether certain genomic regions (genotypes) are associated with disease resistance (phenotypes) and, if yes, then how much does each region account for. The regions that contain one or more disease resistance gene are known as a quantitative trait loci or QTL.

Two approaches can be used in genome-wide QTL scans. The difference between the two lies in how the resource populations are produced, which also influences the density and, consequently, number of genetic markers required.

Linkage Analysis

In this approach, for each marker, one tests whether the inheritance of alleles influences disease resistance in a defined population. This type of linkage is known as identity by descent (IBD).

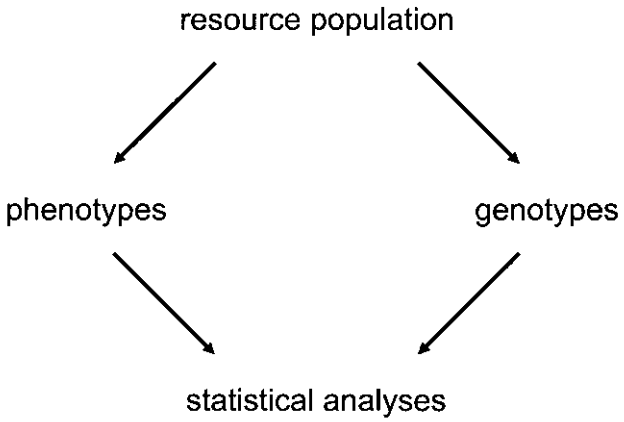


Fig. 2.6. General scheme for identifying genes or genomic regions associated with phenotypes. Using a resource population that is segregating for measurable traits, statistical analyses are used to associate genetic variation with the observed phenotypic variation.

Parents that differ markedly in disease resistance are selected and mated to produce progeny, which favors the use of inbred or divergently selected experimental lines due to their genetic simplicity and defined disease status. In initial QTL scans, the mating structures are often backcross (BC) or F_2 populations. Due to the limited number of generations and recombination events that can erode the amount of LD, marker spacing can be relatively high at 20–40 cM apart, which reduces the number and cost of markers that need to be genotyped.

To validate and fine-map a QTL, additional generations are required to reduce LD and break apart distant marker-disease gene linkages through recombination, leaving only markers that are tightly linked to the disease resistance gene. A popular approach, known as advanced intercross lines (AILs), is to intermate progeny to get F_3, F_4, F_5, etc. (28). With each passing generation, the extent of LD grows smaller, which increases QTL resolving power.

Although simple in concept, the actual execution of the experiment entails a number of factors. Probably the biggest factor that one can control is the number of progeny. The ability to detect QTL with smaller effects increases with more progeny. From a practical standpoint, this means at least 200 birds, if not 1000 or more. Normally, the generation and measurements of resource populations is the rate-limiting step.

A typical result is that a few to 15 QTL are revealed. These are classified as suggestive or significant based on statistical analyses that are corrected for the large number of multiple tests. The reason for including suggestive QTL at this point is that subsequent studies are usually designed to validate potential QTL detected in the first-phase studies. Furthermore, it allows for comparisons across related studies conducted under varied conditions.

How robust is this experimental approach? No one really knows as the true architecture of any one complex trait has not been completely deciphered. In the most advanced study to date (86), after examining 101 traits in 1904 F_{50} mice with 13,549 genetic markers, there was a surprising consistency in genetic architecture across all traits. For most traits, a relatively large number of QTL that explained 1 to 5% of the total phenotypic variation for each trait were identified. And the QTL in total explained around 75% of the genetic variance of each trait. This indicates that it should be possible to achieve a great understanding of disease resistance in the chicken, given powerful enough populations (and funds to perform these tasks). Thus said, even in the powerful mouse study, each QTL was only resolved to a 2 cM interval, which still encompasses 25–50 genes, illustrating the great challenge in going from a QTL region down to a verification of a single gene's effect.

Association Mapping

In association or LD mapping, a pedigreed population is not required, which makes it attractive for use in the commercial environment as existing populations can be tested. The power of this approach is that it relies on historical recombination events to greatly minimize the extent of LD. And rather than following the inheritance of specific alleles (identify by descent), association mapping analyzes genetic marker allele frequency or identity by state (IBS). Thus, resistance birds should be highly enriched for a specific marker allele while the other allele(s) is found in the susceptible birds. This method has both advantages and disadvantages. On the positive side, given the tight linkage required to detect a disease-resistance gene, any genetic marker with a significant association will be relatively close, which means that it is almost immediately amenable to further verification and implementation in a breeding program. On the other hand, since LD is small, one requires a much larger number of genetic markers so that each region is screened.

Association mapping has not been attempted in poultry mainly due to the lack of genetic markers, cost of genotyping, and not having estimates on LD in commercial populations. This situation though has and will continue to change. The identification of nearly 3 million single nucleotide polymorphisms (SNPs) (91) and high-throughput genotyping platforms makes this approach much more feasible.

Genomic Tools

The field of genetics and genomics is heavily influenced by technological advances. Key among these developments are molecular markers, which allow for the generation of high-density genetic maps and the chicken genome sequence. In short, comprehensive genetic maps and the genome sequence allow for the genetic dissection of simplex and complex traits.

Molecular Markers. The beginning of genomics was marked with the development of genetic maps composed of molecular markers. Unlike classical markers, molecular markers are typically based on DNA. Using an assay to type DNA variation, alleles can be determined for each individual, which allows for the development of linkage maps and association studies.

The most commonly used molecular markers are called microsatellites and SNPs. Both of these types of markers rely on specific and unique sequences and can be anchored to a sequence tagged site (STS). Consequently, both genetic and physical distance can be determined for any pair of these molecular markers.

Microsatellites are sequence stretches that contain repeat units of 1 to 6 bases, e.g., CACACACACACACACA or $(CA)_8$. The number of repeat units often varies among individuals. Consequently, PCR primers that amplify this region will produce products that differ in size. The use of automatic DNA sequencers and fluorescently labeled PCR primers allows for the rapid identification of each allele. On average, there is one microsatellite for every 75 kb in the chicken genome. In the chicken genome, 1 cM of genetic distance equates to ~250 kb of physical distance, although this varies substantially across chromosomes. Therefore, there could be several informative and multi-allele microsatellites for every cM in the chicken genome.

SNPs, or single nucleotide polymorphisms, are sequence variants at a specific base in the DNA sequence. In theory, SNPs should have 4 alleles as there are 4 bases (A, C, G, and T). However, most SNPs have only 2 alleles and are often referred to as bi-allelic markers. SNPs are much more frequent than other molecular markers with ~5 found for every kb of chicken sequence. The presence of only 2 alleles greatly simplifies genetic analyses, which makes platforms based on this type of marker the current method of choice for large-scale studies. On the other hand, SNPs are not as informative as microsatellites as they are limited to 2 alleles while microsatellites can have several.

Chicken Genome Assembly. The chicken genome contains 38 autosomes and the Z and W sex chromosomes. In 2002, the NIH funded the sequencing of the entire chicken genome, which was released in 2004. To generate the draft genome sequence assembly, a framework was built using the genetic map as its foundation. Layered on this was a physical map derived from 180,000 BAC (large insert) clones ordered through restriction enzyme analyses. Connections between the genetic and physical maps were made through genetic markers located on individual BAC clones. Finally, the whole-genome shotgun sequence was assembled into contigs and located onto the framework through common sequences; the BAC clones had their ends sequenced. As a result, the initial assembly contained 1.05 Gb of assembled sequence of which 933 Mb were localized to specific chromosomes; the haploid content of the chicken genome is ~1.2 Gb. Each base was sequenced ~6.6 times on average, which is an indicator of genome coverage.

Like all other sequences including the human, there were gaps and assembly errors. In 2006, a second build that incorporated more genetic markers and sequence was generated. Additional sequencing of targeted regions is still under way.

Useful websites on the chicken genome can be found at:

NCBI: http://www.ncbi.nlm.nih.gov/genome/guide/chicken/
UCSC: http://genome.ucsc.edu/cgi-bin/hgGateway
Ensembl: http://www.ensembl.org/Gallus_gallus/index.html

Functional Genomics

As described previously, genetics relies on statistical association of genotypic variation with phenotypic variation to identify genomic regions and determine how much variation each region contributes. Consequently, the ability to identify a disease resistance gene relies on statistical power and probabilities. Currently, this ability translates into QTL being localized to 5 or 20 cM intervals, at best. Even the best mapping studies in mice, with very large populations and dense marker placement, can only place a QTL of moderate effect to a 2 cM interval. This means that it will be extremely difficult to resolve the location of a QTL down to the single gene level. As the chicken genome contains 400 kb per cM and 5 or more genes, on average for the larger chromosomes (43), even a 2 cM interval contains a lot of DNA sequence and candidate genes.

To complement this genetic approach, there are a number of tools that query at the RNA, protein, or metabolite level. These functional genomic tools strive to identify components that vary between two or more states, for example, gene transcripts that are differentially expressed between disease resistant and susceptible chickens. The hope is that by identifying these molecules, gene function and biological pathways can be ascribed to every gene.

Functional genomic assays identify gene transcripts, proteins, or other molecules, which combined with genome-wide QTL scans can reveal positional candidate genes. This integrative approach will be described in more detail for Marek's disease resistance. Below are a few enabling technologies, which can also be applied in a number of ways beyond genetics.

DNA Microarrays

During the generation of whole genome sequences, it became obvious to scientists that there was a huge challenge to study all the genes and gene products that would be identified. Furthermore, it was clear that the reductionist approach of studying one gene or protein at a time would not be sufficient to meet this requirement.

In one of the key technological developments, Pat Brown and colleagues developed DNA microarrays (80). For many years, scientists had used hybridization, which detects complementary sequences for DNA and RNA analysis of specific sequences using labeled probes. In an elegant extension of this concept, all the probe sequences instead of being individually labeled were arrayed onto a microscope slide and the total mRNA population of the tested individual was labeled. As a consequence, the relative expression of thousands of genes in a single sample could be simultaneously measured in one step.

Within a relatively short period of time, this technology was adapted for all species that had substantial RNA and DNA sequence information or resources. With respect to chicken, Affymetrix presently makes the most comprehensive microarray with coverage extending to all the ~28,000 genes predicted in the first genome assembly. In addition, there are probes sets for genes to 17 different avian viruses including Marek's disease virus, Newcastle disease virus, and avian influenza, which allows this tool to be used for veterinary diagnostics in the detection of these viruses.

DNA microarray experiments generate a list of genes that are expressed more or less between two or more samples. For example, genes A, B, and C may be expressed more and genes X, Y, and Z less in the thymus of 5 day old resistant birds compared to age-matched susceptible birds. It is important to note that because

gene expression (RNA) is being measured, unlike DNA, it can vary depending on the bird, tissue, time point, and other influences. Because of this natural biological variation, even for a given set of conditions, it is critical that several replicates are conducted. Unfortunately, at the moment, this is a relatively expensive procedure as each sample can cost $500 or more to process, which when combined with more variables (e.g., several tissues, more time points) inflates the total experiment cost quickly.

The list of genes can be further analyzed to provide a higher order understanding. For example, if all the genes belonging to a specific biological or functional class are elevated in the resistant bird compared to the susceptible bird, then most likely this pathway is involved in disease resistance. Tools to better analyze and extend the biological meaning of array results is an area of intense study.

With the success of this highly parallel process, it was quickly adopted to many other molecules or situations. There are now protein arrays, tissue arrays, metabolite arrays, with the list going on and on. Undoubtedly, many of these techniques will become viable and implemented in poultry in the near future. For reviews on diagnostic applications in animals, see references 33, 81, 89.

Proteomics

Moderate to large scale global screening at the protein level is also available today. For identification of proteins in a complex mixture, mass spectrometry is the technique of choice (29). Typically, protein samples are fractioned to reduce the complexity; then peptides are analyzed by the mass spectrometer; and finally the data sets are analyzed to deduce the identity of proteins. Although simple in concept, analysis of proteins is much more challenging than either DNA or RNA because for each gene, there are many proteins with varying post-translational modifications, and the concentration of proteins encompasses many orders of magnitude. Consequently, it is fairly simple to identify the abundant proteins but difficult for the ones in low quantity. And like DNA microarrays, cost can be a serious issue, which partially explains the minimal usage of this technique thus far in poultry research, though the situation is gradually changing (20).

Besides identifying individual proteins, it is possible to screen for protein-protein interactions. The classic method, known as the two-hybrid screen (34, 35), takes advantage of the ability to reconstitute the activity of a transcription factor that is in two parts. By fusing a cDNA (prey) library to the activation domain (AD) of a transcriptional activator, it is possible to identify proteins that interact with bait (protein of interest) that is fused with the DNA-binding domain (BD). As the AD and BD do not need to be physically connected to promote transcription, if two proteins interact, a reporter gene is expressed if AD and BD are brought into close proximity of each other. Due to the nature of the assay, all interactions must be independently confirmed to eliminate false positives.

Higher order interactions can be revealed with gentle cell lysis and immunoprecipitation of a protein complex using antibodies directed against one member. The identity of the other interacting proteins can be quickly revealed through mass spectrometry.

Genetic Resistance to Specific Diseases

As discussed previously, one generally needs to first demonstrate heritability of resistance to disease to demonstrate a genetic component. Although differences for disease resistance between chicken genetic lines may suggest a genetic basis, they are insufficient to prove it. Combined with the need in molecular genetics for controlled disease challenges and marker associations, the number of studies conducted to date is limited, especially for genome-wide scans. There is, however, evidence for genetic control of poultry diseases caused by a wide range of pathogens, including viruses, bacteria and parasites.

Avian Leukosis

As described in Chapter 15, avian leukosis viruses (ALV) are a group of retroviruses that can induce tumors. ALVs are classified into various subgroups based on virus-specific cellular receptors and virus envelope glycoproteins. The subgroups that infect chicken are A–E and J with all but subgroup J being exogenous ALVs.

Genetic resistance to ALVs subgroups A–E is well defined and based on specific cellular receptors. Since a single functioning receptor allele is all that is required for virus entry, susceptibility is dominant and the genetics is simple with only one locus involved. Molecular studies taking advantage of this fact have revealed not only the encoding gene but the basis for differences between resistant and susceptible lines. Interestingly, although the ALV subgroups are related and thought to have arisen from a common ancestor, all the cellular receptors show no obvious sequence or structural similarity.

Resistance to ALV subgroup A is determined by the tva locus found on chromosome 28, and encodes a protein a member of the low-density lipoprotein receptor (LDLR) family of unknown function (10, 30). There have been two alleles identified that confer resistance. The tva^r allele contains a single nucleotide mutation that results in a protein with very low binding affinity to the ALV subgroup A envelop while the other resistance allele, tva^{r2}, has a 4-nucleotide insertion near the beginning of the coding sequencing, which results in an altered protein.

The *tvc* locus, which confers resistance to ALV subgroup C, is ~1 cM from *tva* (31). This receptor shows homology to butyrophilins, a member of the immunoglobulin superfamily. The resistant allele contains a premature stop codon and, thus, would not produce a complete and functioning receptor (32).

Resistance to ALV subgroups B, D, and E (endogenous) are all controlled by the *tvb* locus (2), which is located on chromosome 22 (82). There are several reported alleles for this receptor, which is related to the tumor necrosis factor receptor (TNFR) family. A single nucleotide change that generates a premature stop codon, results in a non-functioning receptor and resistance to all three subgroups. The tvb^{s1} or wild-type allele is susceptible to all three subgroups while the tvb^{s3} allele confers resistance to subgroup E as the result of a different single nucleotide mutation (53). Molecular tests that can determine the tvb genotype have been reported (97).

Although the receptor for ALV subgroup J has been identified and shown to be Na+/H+ exchanger 1 (NHE1) (22), no cellular resistance has been observed, which suggests that variants that do not allow for virus binding are lethal. Field reports showing differences between lines suggest a genetic basis for differences in resistance to the myeloid leukosis.

Marek's Disease

Chickens resistant to Marek's disease (MD) are those that fail to develop characteristic symptoms upon exposure to MDV (see Chapter 15). Genetic differences in resistance to fowl paralysis, assumed to be MD, have been reported for 60 years (3). Resistance to MD is complex and controlled by multiple genes or QTLs (6).

The best understood mechanism for the involvement of genetic resistance to MD involves the major histocompatibility complex (MHC) or, as it is known in the chicken, the *B* complex. The MHC contains three tightly linked regions known as *B–F* (class I), *B-G* (class II), and *B-L* (class IV), which control cell surface antigens. The *B-G* locus is expressed in erythrocytes, which enables convenient typing of blood groups. By measuring the allelic frequency of specific blood groups, it has been observed that certain B alleles are associated with resistance or susceptibility. Chickens with the B^{21} allele have been found to be more resistant than those with other *B* haplotypes (4, 8). (Haplotype refers to a set of alleles that is frequently co-inherited.) Other studies have allowed for the relative ranking of the other B alleles: moderate resistance, B^2, B^6, B^{14}; susceptibility, B^1, B^3, B^5, B^{13}, B^{15}, B^{19}, B^{27} (67) The MHC also influences vaccinal immunity as some haplotypes develop better protection with vaccines of one serotype than of a different serotype (7, 9).

In addition to the MHC, chromosome 16 contains another set of class I and class II MHC genes that is called the *Rfp-Y* locus (73). Work by Wakenell (90) using data obtained from commercial chickens challenged with MDV suggests an association with MD resistance. However, results from matings with experimental lines indicate that the *Rfp-Y* genes do not influence any MD associated trait in these genetic backgrounds (88). These conflicting results further demonstrate the complexity of genetic resistance to MD, and the probable influence of genetic background.

Besides the MHC, other genetic factors have a major influence on resistance to MD. For example, lines 6 and 7 chickens share the same *B* haplotype, B^2, yet are MD resistant and susceptible, respectively. Due to the availability of these characterized lines and their genetic simplicity, genome-wide QTL scans have been conducted. In one study, unvaccinated F_2 progeny were challenged with MDV and measured for MD as well as a variety of MD-associated traits such as viral titer, number of tumors, and length of survival (87, 92). Using genetic markers spaced throughout the chicken genome, 14 QTL (7 significant and 7 suggestive) were discovered that explain one or more MD-associated traits. The QTL were of small to moderate effect as they explained 2 to 10% of the variance, with additive gene substitution effects from 0.01 to 1.05 phenotypic standard deviations. Collectively, the QTL explained up to 75% of the genetic variance. Interestingly, 10 of the 14 QTL displayed non-additive gene action; 3 with overdominance (individuals that are heterozygous have a phenotype better adapted than either class of homozygous individual) and 7 were recessive. With multiple traits being measured, the QTL could be grouped. In the first set, 3 of the QTL were associated almost exclusively with viremia levels while the remaining QTL could account for disease, survival, tumors, nerve enlargement, and other disease associated traits. This suggests that disease resistance occurs at least at two levels: initial viral replication and cellular transformation, which occurs later. It also highlights the added value of measuring several components as it may functionally separate a complex trait as well as provide clues on positional candidate genes.

In the second study, Bumstead (17) used a (6 x 7) x 7 backcross population and measured MDV replication by quantitative polymerase chain reaction (qPCR). A single large effect QTL on chromosome 1 was identified, which accounted for approximately half of the viral replication differences between the two parental lines. This result also supports the relationship between virus titers and disease resistance.

As described previously, going from a QTL to identification of the underlying causative genes is a tremendous challenge, even in model organisms. Consequently, additional functional genomic approaches have been incorporated to give further screens. The first approach utilizes DNA microarrays to profile transcripts that are differentially expressed between MD resistant or susceptible lines. Gene profiling has been conducted to identify differentially expressed genes between lines 6 and 7 after MDV challenge (61), among *B* (MHC) congenic lines of chicken following inoculation with different MD vaccines, and in chicken embryo fibroblasts (CEF) infected with MDV (74). Analyses of these experiments have identified a number of genes and pathways that are consistently associated with either MD resistance or MDV infection. More importantly, the results suggest that chickens with immune systems that are more stimulated by MDV infection are more susceptible. Initially, this seems counterintuitive but upon further reflection, MDV is thought to only infect activated lymphocytes and, thus, chickens with immune systems that are more responsive may present more targets for MDV to infect and later transform.

The second approach uses the two-hybrid assay to identify host proteins that interact with specific MDV proteins. Thus far, 9 MDV-chicken protein-protein interactions have been confirmed by an in vitro binding assay, which demonstrates that the interactions are direct and specific, and do not require other intermediary factors (e.g., yeast proteins) to be involved (76).

In both approaches, a finite number of candidate genes are obtained, which can be further evaluated for a genetic influence. For example, having confirmed the MDV SORF2-chicken growth hormone (GH) interaction, the GH gene (*GH1*) was evaluated as a candidate gene for MD resistance. *GH1* variation was significantly associated ($P<0.01$) with a number of MD-associated traits in MHC B^2/B^{15} chicks from commercial White Leghorn lines (62). Furthermore, DNA microarray results indicate that GH is differentially expressed between MD resistant (line 6) and susceptible (line 7) chicks following MDV challenge (61). Thus, the combined results of a specific MDV-chicken protein interaction, differential expression of GH between MD resistant and

susceptible chickens, and association of *GH1* with MD disease-related traits and selected lines for MD resistance, all strongly suggest that *GH1* is a MD resistance gene (62). Using the same strategy, *LY6E* [lymphocyte complex 6, locus E, aka, stem cell antigen 2 (*SCA2*) and thymic shared antigen 1 (*TSA1*)] (63) and *BLB*, the gene for MHC class II β chain (76), meet the same criteria.

Salmonellosis

Control of salmonellosis presents special challenges to the poultry industry. As reviewed in detail elsewhere in this book (Chapter 16), some species of *Salmonella* bacteria are highly pathogenic and contagious in chickens, while other species cause little response in the host birds, which can then become asymptomatic carriers. Because they are maintained in the production flocks, birds with subclinical salmonellosis can transmit zoonotic bacteria into the human food chain. Chicks infected with *Salmonella* immediately after hatch can be persistently colonized to maturity, when the bacteria are shed vertically to infect table or hatching eggs, or horizontally to infect other hens (36).

Heritability estimates of various parameters of *Salmonella* response indicate that genetic selection to improve resistance to salmonellosis is feasible. The heritability of chick mortality after *Salmonella* challenge was estimated at 0.14 and 0.62 for sire and dam components, respectively (11). Estimated heritability of resistance to cecal carrier state, measured by enrichment culture, in laying hens was 0.20 (13). Heritabilities of number of bacteria persisting in internal organs ranged from 0.02 to 0.29 (39). Estimated heritability of antibody response to *Salmonella* ranged widely, from 0.03 to 0.26 (11, 52). Measuring antibody response is less expensive and laborious than measuring bacterial colonization and because vaccine antibody has a negative genetic correlation with cecal colonization, vaccine antibody is a useful biomarker to improve resistance to colonization (50).

Many genes are associated with genetic control of response to *Salmonella* species. The individual effect of most genes on the disease phenotypic variation is relatively small, often only 3 to 5% of the total variation, which is consistent with complex control of the disease by many genes. Important factors, such as chicken genetic line, population structure, definition of response (antibody, mortality, systemic or enteric colonization), and serovar of *Salmonella* differed among studies, resulting in some variation in results. However, a generally consistent picture of genetic control has emerged.

The strategy of comparative genomics was very effectively used to identify genes controlling salmonellosis resistance in chickens because response to *Salmonella* was previously studied in detail in the mouse, a model organism. Chicken homologs of major loci controlling natural resistance of mice to infection with *S. typhimurium* were examined as candidate genes. Together, *NRAMP1* (natural resistance-associated macrophage protein 1, now called SLC11A1 for solute carrier family 11 member 1) and *TNC* (a locus closely linked to *LPS*, now known as *TLR4*, which binds lipopolysaccharide, a major component of Gram-negative bacteria membranes) accounted for 33% of the differential resistance in *Salmonella*-induced mortality in a backcross population of inbred lines (44). Subsequently, the *NRAMP1* association was confirmed for several parameters of host response to *Salmonella* in other chicken populations (12, 54, 64), as was the *TLR4* association (12, 57, 68).

The success of the comparative genomics approach provided a starting point to select additional candidate genes to test. Some genes were positional candidates based upon genomic position, such as *CD28* and *VIL1* in the *NRAMP1* region (38). The *CD28* gene was associated with enteric *Salmonella* infection (68), and *VIL1* with visceral infection (39).

Other candidate genes were selected based upon involvement in pathways that are hypothesized to be important in host response to *Salmonella*. The product of the *MD2* gene interacts with the *TLR4* receptor on the cell surface, and SNPs in *MD2* are associated with persistence of *Salmonella* colonization in the cecum (68). The MHC, because of its crucial role in antigen processing and presentation, was investigated. In a series of 12 B-complex congenic lines, line differences occurred in morbidity and mortality after *Salmonella* challenge (26). The MHC class I was associated with resistance to *Salmonella* colonization in the spleen in an experimental cross (66). Genes in apoptotic pathways include *CASP1* and *IAP1*. A *CASP1* SNP was associated with *Salmonella* persistence in the spleen and cecum in an experimental cross (65) and in the liver and cecum in commercial broilers (54). The *IAP1* gene was associated with spleen (65) and cecum bacterial load (54). The genomic region containing genes encoding a newly identified family of antimicrobial peptides, the gallinacins, was associated with *Salmonella* vaccine antibody response (41).

Although the design of most candidate gene experiments does not preclude that the causal gene could be a nearby gene rather than the specific one studied, having supporting lines of evidence such as confirmation in independent populations, with QTL scans, by gene expression data, or from comparative genomics, helps to add confidence in the detected gene-resistance associations. Studies on gene expression in *Salmonella* challenged versus unchallenged, or resistant versus susceptible animals, are revealing differential expression in genes that may be active in pathways controlling resistance (23, 49, 98).

Genome-scan studies have identified QTL regions controlling *Salmonella* resistance. In analysis of a backcross population of resistant and susceptible parental inbred lines (lines 6_1 and 15I, respectively), genotyping of animals with extreme bacterial counts in spleen showed significant linkage between markers on chromosome 5 and the resistance trait (70). Fine-mapping of the chromosomal location of the QTL found a very strong effect, accounting for 50% of the parental variation difference, in the region near the *CKB* and *DNCH1* genes. The specific gene associated with resistance is not positively identified, however, and the *Salmonella* resistance QTL in this region is considered a novel locus named *SAL1*. The distance of almost 50 cM between *SAL1* and the *Salmonella* resistance QTL linked to marker ADL0298 (51) makes them unlikely to represent the same locus although both influenced bacterial colonization in the spleen.

Another genome scan for *Salmonella* resistance QTL used F_2 and BC populations formed from line N and 6_1, and assessment

of cloacal and cecal carrier state (84). This study confirmed an association of the *SAL1* region with enteric carrier state. Additionally, new QTL regions were identified at significant or suggestive levels on chromosomes 1, 2, 5, and 16, with some having effects as large as 37.5% of the phenotypic variance (84). The QTL on chromosome 16 lies in the MHC, although the two inbred lines are considered to have the same MHC haplotype. The QTL on chromosome 5 maps near *TGFB3*, which was shown in independent populations to be associated with spleen (54) and cecal colonization (69).

Colibacillosis

Escherichia coli is an important pathogen of poultry, both as a cause of colibacillosis in production flocks and as a potential food-safety pathogen (Chapter 18). Elucidation of the genetic control of resistance to *E. coli* has primarily been approached through analysis of lines that were divergently selected for circulating antibody level to *E. coli* vaccine in young birds. The principle underlying this approach is that if vaccine antibody is related to resistance and if resistance is under genetic control, then the genetic selection for antibody response will generate lines that differ in resistance genetics. These lines are then a resource to identify the specific genetic differences controlling resistance.

Divergent selection for *E. coli* vaccine antibody response successfully modulated mortality rate, and the immune response to pathogenic *E. coli* and several other antigens (42). Genetic control of *E. coli* response was studied in an F_2 population generated from crossing the divergently selected lines. Molecular probes were used for three candidate genes in the MHC region: *B-F, B-G* and *TAP2* (94). Each probe revealed genetic variation in the population (assessed by RFLP bands), and each gene had multiple bands associated with antibody traits. Also, analyzing the effect of all probes simultaneously revealed more significant effects than analyzing each separately.

Genome scans of resource populations produced from the same divergently selected lines revealed QTL for antibody response and mortality. The initial low-density scan with 25 markers found three markers associated with antibody response to *E. coli*, Newcastle disease virus, and/or SRBC and one with mortality (93). In a more extensive study, F_1 males derived from a cross of the divergently selected lines were mated with females of four genetic backgrounds (the F_1, the two divergent lines, and a commercial line) to produce 1700 progeny that were immunized with *E. coli* and *Salmonella enteritidis* vaccine (95). Individuals with the highest or lowest average antibody were selectively genotyped within each mating type and sire family. About 125 markers were heterozygous in each sire. Twelve markers were associated with QTL for *E. coli* antibody, and six of these were also associated with *S. enteritidis* antibody, with two markers suggesting a QTL on chromosome 2. The experimental design, with sires mated to dams of multiple lines, also demonstrated that the effect of markers was modulated by the genetic backround.

Coccidiosis

Coccidiosis is caused by several species of parasites of the genus *Eimeria*, each of which has tropism for a different area of the gastrointestinal tract (Chapter 7). The cost of control of coccidiosis by pharmaceutical means, continual emergence of drug-resistant strains, and profit loss due to reduced growth efficiency all mandate the need for other approaches, such as genetic resistance, to control this disease. Population differences, and the ability to genetically select lines for divergent resistance/susceptibility, illustrate the feasibility of improvement in genetic resistance to coccidiosis (15, 46, 77).

Blood group antigens comprise the major category of genes associated with coccidiosis resistance, with the well-characterized B locus, or MHC, representing most of the reported associations. Several studies compared MHC (or B)-congenic lines, which share background genes but differ for the B locus region (21, 58, 79), or populations in which MHC haplotypes segregate within line or differ among lines (14, 24, 72, 85). Collectively, these studies provide strong support for the MHC being a coccidiosis resistance locus. However, effects of specific MHC haplotypes greatly vary, dependent upon factors such as *Eimeria* challenge strain and the specific trait used to characterize resistance (for example, antibody, oocyst shedding, weight gain, severity of intestinal lesions). A study of birds that were recombinants between the B–F and B–G (class I and II, respectively) regions of the MHC suggested that the MHC class I region had the greater role in resistance (25). Although studies of MHC-congenic lines reveal a clear picture regarding the role of the specific MHC haplotypes studied, they do not shed light on what impact genomic variation at other regions might have on resistance. Experiments that included variation in both the MHC haplotypes and the genetic background determined that both MHC and non-MHC genes control resistance to coccidiosis (59, 60). Erythrocyte loci (identified serologically) in addition to the B locus have been associated with resistance to coccidiosis, including *Ea-A, Ea-E* (46), *Ea-C* (47), and *Ea-I* (71).

An F_2 cross of commercial broiler lines was used to conduct a genome scan for QTL associated with resistance to coccidiosis caused by *Eimeria maxima* (99). Using 119 markers that gave about 80% genome coverage, a strong QTL for oocyst shedding was located on chromosome 1.

Infectious Bursal Disease

Infectious bursal disease virus (IBDV) causes acute infection and depletion of B cells in the bursa and other organs, often resulting in severe and permanent immunosuppression of the host (Chapter 4). Mating of resistant and susceptible lines to form F_1, F_2 and BC populations demonstrated IBDV-induced mortality to be under the control of a fully or partially dominant autosomal resistance gene (18, 19). Multiple studies have examined the MHC for association with IBDV resistance and detected no effect (18, 19, 45). In one study, however, an MHC effect was detected on resistance parameters of specific antibody to IBDV and bursal histopathology (48). The differences among studies are likely to be a result of examination of different MHC haplotypes in varied genetic backgrounds. Collectively, studies on genetics of IBDV resistance suggest involvement of the MHC and at least one other, currently unidentified, autosomal gene. Global transcriptional profiling has revealed numerous genes that are differentially regulated between

IBDV resistant and susceptible lines, and suggests that resistance may be mediated by more rapid inflammatory response and more extensive p53-related apoptosis of target B cells, thus limiting viral replication in resistant birds (78).

Future Perspectives

Genetic resistance to disease for any one individual or line of chickens is the overall outcome of a very complex set of signals and responses with which the chicken and pathogen interact. The ability for molecular genetics to unravel this biological complexity has been very impressive. Despite the rapid pace of advancements, there is a long way to go before scientists develop a detailed understanding of the molecular pathways, and poultry breeders are able to transfer this information to improve their commercial products.

Poultry breeding companies have achieved enhanced disease resistance in their flocks to many pathogens using traditional methods. However, this is a laborious process that requires the intentional exposure of individuals from elite lines to pathogens, and progresses rather slowly due to indirect selection on overall livability. With the emergence of molecular genetics and the field of genomics, there is a optimism that studies will be able to identify genes and alleles that confer superior disease resistance. When applied to breeding, the information will provide for the rapid and accurate improvement of commercial lines. In application, the poultry breeders do not necessarily rely on the identification of the disease resistance gene itself, but may use markers in linkage disequilibrium to increase the frequency of favorable alleles. But as science has shown many times, increasing knowledge gives increasing power that can often be applied in novel ways. So the ultimate goal should be to identify disease resistance genes and their pathways to reveal biological function and pathways.

So what will hasten the advancement of knowledge? With high-throughput platforms to determine genotypes, the rate-limiting step is in producing and measuring resource populations. With respect to animal production, one solution would be to increase the partnerships between poultry breeding companies and molecular geneticists, which allows for the leveraging of expertise and resources. This partnership also allows for scientists to work at the population level with all the diverse set of genes and allele combinations. Beyond this, trait measurements will be critical. As DNA microarrays and proteomics have already shown, breaking down a trait into specific components provides critical information on biological processes that cannot be or are difficult to obtain by traditional phenotypic measurements. Thus, veterinary medicine can make key contributions by refining trait measurements associated with disease and disease progression.

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Chapter 3

Newcastle Disease, Other Avian Paramyxoviruses, and Pneumovirus Infections

Introduction

D. J. Alexander and D. A. Senne

The virus families *Paramyxoviridae, Filoviridae,* and *Rhabdoviridae* form the virus order *Mononegavirales* (i.e., the single-stranded, nonsegmented, negative-sense RNA viruses showing helical capsid symmetry). The family *Paramyxoviridae* is divided into two subfamilies *Paramyxovirinae* and *Pneumovirinae* (4).

The subfamily *Paramyxovirinae* has five genera: *Rubulavirus,* which includes the mumps virus, mammalian parainfluenzas 2 and 4; *Respirovirus* containing mammalian parainfluenza viruses 1 and 3; *Morbillivirus,* which includes measles, distemper, and rinderpest; *Henipavirus* formed from the Nipah and Hendra viruses; and the *Avulavirus* genus formed from Newcastle disease virus (NDV), and other avian paramyxoviruses (4).

Nine serogroups of avian paramyxoviruses have been recognized: APMV-1 to APMV-9 (1). Of these, Newcastle disease virus (APMV-1) remains the most important pathogen for poultry, but APMV-2, APMV-3, APMV-6, and APMV-7 are known to cause disease in poultry. The prototype viruses and the recognized natural hosts for each serogroup are shown in Table 3.1. Detailed descriptions of serotypes not shown to affect poultry and serotypes usually infecting feral waterfowl have been reviewed by Alexander (1–3).

The subfamily *Pneumovirinae* has two genera: *Pneumovirus,* consisting of mammalian pneumoviruses, and *Metapneumovirus.* Avian viruses of this genus are correctly referred to as "avian metapneumoviruses" and disease in turkeys is referred to as "avian metapneumovirus infection of turkeys." However, avian pneumovirus[es] and turkey rhinotracheitis virus [TRTV] are still widely used.

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Newcastle Disease

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Introduction

Newcastle disease virus varies widely in the type and severity of the disease it produces. This variety has often caused some problems in recognizing the disease as Newcastle disease (ND) when it is introduced into a country or area and consequently with nomenclature. ND is particularly complicated in that different isolates and strains of the virus may induce enormous variation in the severity of disease, even in a given host such as the chicken. To simplify matters, division into forms or pathotypes of disease based on clinical signs in chickens has been made as summarized by Beard and Hanson (38): 1) Doyle's form (77), which is an acute, lethal infection of all ages of chickens. Hemorrhagic lesions of the digestive tract are frequently present, and this form of disease has been termed viscerotropic velogenic Newcastle disease (VVND). 2) Beach's form (34), which is an acute, often lethal infection of chickens of all ages. Characteristically, respiratory and neurological signs are seen, hence the term neurotropic velogenic (NVND). 3) Beaudette's form (41) that appears to be a less pathogenic form of NVND in which deaths usually are seen only in young birds. Viruses causing this type of infection are of the mesogenic pathotype and

Table 3.1. Prototype viruses and host range of avian paramyxoviruses.

Prototype virus strain	Usual natural hosts	Other hosts	Disease produced in poultry
APMV-1—Newcastle disease virus	Numerous	See text	Varies from extremely pathogenic to inapparent, depending on strain and host infected
APMV-2/chicken/California/Yucaipa/56	Turkeys, passerines	Chickens, psittacines, rails	Mild respiratory disease or egg production problems, severe if exacerbation occurs
(1) APMV-3*/turkey/Wisconsin/68	Turkeys	None	Mild respiratory disease but severe egg production problems worsened by exacerbating organisms or environment
(2) APMV3*/parakeet/Netherlands/449/75	Psittacines, passerines	None known	None known
APMV-4/duck/Hong Kong/D3/75	Ducks	Geese	None known
APMV-5/budgerigar/Japan/Kunitachi/74	Budgerigars	None known	No infections of poultry reported
APMV-6/duck/Hong Kong/199/77	Ducks	Geese, rails, turkeys	Mild respiratory disease and slightly elevated mortality in turkeys; none in ducks or geese
APMV-7/dove/Tennessee/4/75	Pigeons, doves	Turkeys, ostriches	Mild respiratory disease in turkeys
APMV-8/goose/Delaware/1053/76	Ducks and geese	None known	No infection of poultry reported
APMV-9/domestic duck/New York/22/78	Ducks	None known	Inapparent infection of commercial ducks

*Serological tests may distinguish between turkey and psittacine isolates.

have been used as secondary live vaccines. 4) Hitchner's form (121), represented by mild or inapparent respiratory infections caused by viruses of the lentogenic pathotype, which are commonly used as live vaccines. 5) Asymptomatic-enteric form (166), which is chiefly a gut infection with lentogenic viruses causing no obvious disease. Some live commercial vaccines are of this pathotype.

Definitions and Synonyms

ND has been termed pseudo-fowl pest, pseudovogel-pest, atypische Geflugelpest, pseudo-poultry plague, avian pest, avian distemper, Ranikhet disease, Tetelo disease, Korean fowl plague, and avian pneumoencephalitis.

The nomenclature may also be confusing as sometimes infection of birds with any strain of the NDV may be termed ND. Strictly, ND should be reserved for infections with those viruses falling within the internationally accepted definition. To avoid confusion, the abbreviation vND will be used in this chapter for the disease caused by the defined virulent strains of virus.

Since the early 1980s there has been a continuing panzootic in pigeons. The strain of vNDV responsible has been termed "pigeon paramyxovirus type 1 (PPMV-1)," although this is for pragmatic purposes, and the virus will cause vND in poultry.

Economic Significance

The global economic impact of vND is enormous. Until the emergence of the highly pathogenic Asian H5N1 influenza virus this impact was unsurpassed by any other poultry virus and probably represented a bigger drain on the world's economy than any other animal virus. In developed countries with established poultry industries, not only are outbreaks of vND extremely costly, but control measures, including vaccination, represent a continuing loss to the industry (158). Even countries free of vND usually face the cost of repeated testing to maintain that status and for the purposes of trade. In many developing countries vND is endemic and, therefore, represents an important limiting factor in the development of commercial poultry production and the establishment of trade links. Many countries rely on village chickens to supply a significant portion of dietary protein in the form of eggs and meat, especially for women and children. The constant losses from vND (227, 234) severely affect the quantity and quality of the food of people on marginal diets. Therefore, the economic impact of vND should not only be measured in direct losses in commerce but in some countries, the effect on human health and loss of potential socioeconomic growth should also be considered.

Public Health Significance

Apart from its contribution to malnutrition, NDV is a recognized human pathogen in its own right. Reports of disease have often been anecdotal but the best substantiated clinical signs in human infections have been eye infections, usually consisting of unilateral or bilateral reddening, excessive lachrymation, edema of the eyelids, conjunctivitis and subconjunctival hemorrhage (60). Infections are usually transient, and the cornea is not affected. There have been less well-substantiated reports that a more generalized infection may sometimes occur resulting in chills, headaches, and fever, with or without conjunctivitis (60). Evidence shows that both vaccinal and virulent [for poultry] strains of NDV may infect and cause clinical signs in humans. Human infections with NDV have usually resulted from direct contact with the virus such as from splashing infective allantoic fluid into

the eye in laboratory accidents; rubbing the eye with hands, etc., contaminated with virus after handling infected birds or their carcasses; and contamination of vaccination personnel especially when vaccines are given by aerosol. Such infections usually can be avoided by basic hygiene and appropriate clothing and eye protection. Casual contact with infected poultry represents a low risk of human infection. No reports exist of human-to-human spread.

History

It is generally considered that the first outbreaks of vND occurred in 1926, in Java, Indonesia (151), and in Newcastle-upon-Tyne, England (77). There are reports of disease outbreaks in Central Europe similar to what we now recognize as vND that predate 1926 (106), and Levine (160), citing Ochi and Hashimoto, indicated that the disease may have been present in Korea as early as 1924. Macpherson (163) considered the death of all the chickens in the Western Isles of Scotland in 1896 to be attributable to vND.

The name "Newcastle disease" was coined by Doyle as a temporary measure because he wanted to avoid a descriptive name that might be confused with other diseases (78). No better name has evolved over the past 75 years, although for the virus the synonym avian paramyxovirus type 1 (APMV-1) has gained some popularity in recent years. In fact, APMV-1 is often used to describe the low virulent strains to avoid using Newcastle disease that, according to definitions used by the World Organization for Animal Health and other international agencies, should be reserved for virulent viruses.

Some years after the 1926 outbreaks and recognition of the virus etiology, it became clear that other less severe diseases were caused by viruses indistinguishable from NDV by conventional methods. In the United States, a relatively mild respiratory disease, often with nervous signs, was first described in the 1930s and subsequently termed *pneumoencephalitis* (34). It was shown to be due to a virus indistinguishable from NDV in serologic tests (35). Within a few years, numerous NDV isolations that produced extremely mild or no disease in chickens were made around the world (29, 121, 166, 228).

The history of Newcastle disease in most countries has not been well-documented. Alexander (11) recorded the history in Great Britain in detail and considered it a good example of the effect that Newcastle disease may have on the poultry industry in a developed Western country.

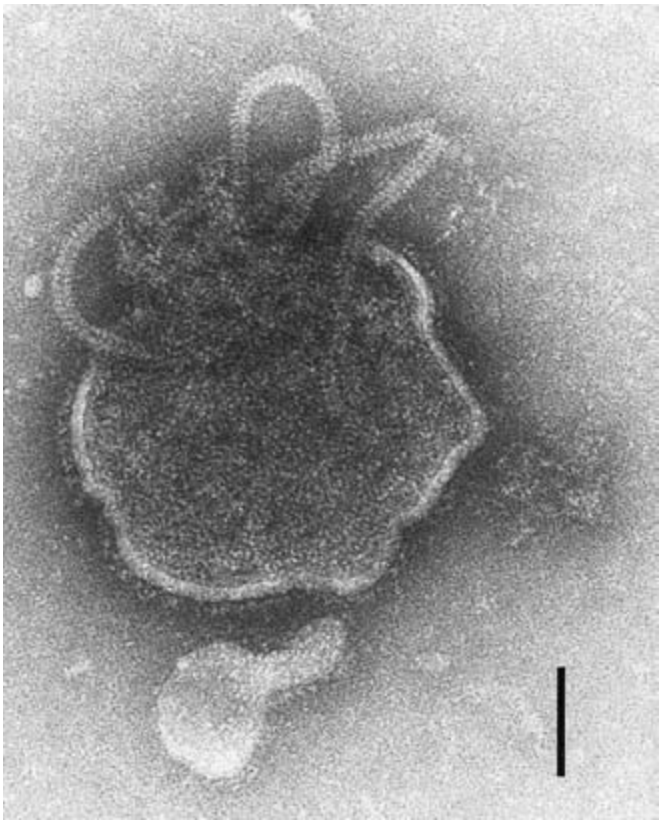
Etiology

Classification

See "Introduction" at the beginning of this chapter.

Morphology

Negative contrast electron microscopy of NDV reveals very pleomorphic virus particles typical of members of the *Paramyxovirinae* subfamily. Generally, they are rounded and 100–500 nm in diameter, although filamentous forms of about 100 nm across and of variable length are often seen. The surface of the virus particle is covered with projections about 8 nm in length. In most electron micrographs, the "herring bone" nucleocapsid, about 18 nm across and showing helical symmetry, may be seen either free or emerging from disrupted virus particles (Fig. 3.1).



3.1. Negative contrast electron micrograph of Newcastle disease virus strain Ulster 2C showing a partially disrupted particle with nucleocapsid emerging. ×202,000, bar = 100 nm. (Collins)

Chemical Composition

Paramyxoviruses characteristically consist of a single molecule of single-stranded RNA of about 5×10^6 molecular weight (148), which makes up about 0.5% by weight of the virus particle. Nucleotide sequencing of the NDV genome has shown it to consist of 15,186 nucleotides (200), although strains with 15,192 (126) and 15,198 (75) nucleotides have been described.

Virus particles have about 20–25% w/w lipid derived from the host cell and about 6% w/w carbohydrate. The overall molecular weight for an average virus particle is about 500×10^6, with a density in sucrose of 1.18–1.20 g/mL.

The six genes forming the genome of NDV code for seven proteins (154) are as follows: L protein is the RNA-directed RNA polymerase associated with the nucleocapsid; HN is responsible for the hemagglutinin and neuraminidase activities, forming the larger of the two types of projections seen on the surface of paramyxovirus particles; F, fusion protein, forms the smaller of the surface projections; NP, nucleocapsid protein; P, phosphory-

lated, nucleocapsid-associated, the P gene has an overlapping reading frame that codes for the cysteine-rich V protein; and M, matrix. The order of the genes for these proteins in the virus genome is 3′NP/V-M-F-HN-L5′. The host protein actin is also incorporated into virus particles.

Biologic Properties

Several biologic properties are associated with paramyxoviruses, which characterize the group.

Hemagglutination Activity

The ability of NDV and other avian paramyxoviruses to agglutinate red blood cells (RBCs) is due to the binding of the hemagglutinin-neuraminidase (HN) protein to receptors on the surface of the RBCs. This property and the specific inhibition of agglutination by antisera (57) have proven to be powerful tools in the diagnosis of the disease.

Chicken RBCs usually are used in hemagglutination (HA) tests, but NDV will cause agglutination of all amphibian, reptilian, and avian cells (156). Winslow *et al.* (260) showed that human, mouse, and guinea pig RBCs were agglutinated by all NDV strains tested, but the ability to agglutinate cattle, goat, sheep, swine, and horse cells varied with the strain of NDV. Other avian paramyxoviruses also appear to be able to agglutinate a wide range of RBCs, but the exact range may vary with isolate as well as serotype. Paramyxoviruses will agglutinate cells other than RBCs if they possess the correct receptors.

Neuraminidase Activity

The enzyme neuraminidase (mucopolysaccharide *N*-acetyl neuraminyl hydrolase EC 3.2.1.18) is also part of the HN molecule. An obvious consequence of the possession of this enzyme is the gradual elution of agglutinated RBCs (3). The exact function of the neuraminidase in virus replication is unknown, but it seems likely that neuraminidase removes virus receptors from the host cell which prevents the reattachment of released virus particles and virus clumping.

Cell Fusion and Hemolysis

NDV and other paramyxoviruses may bring about hemolysis of RBCs or fusion of other cells by essentially the same mechanism. Attachment at the receptor site during replication is followed by fusion of the virus membrane with the cell membrane, which may result in the fusion of two or more cells (similar to the syncytial formation that occurs when virus particles are budded from cells). The rigid membrane of the RBCs usually results in lysis from the virus membrane fusion.

Virus Replication

The strategy for replication employed by NDV is that of the negative strand viruses in general and avulaviruses specifically (154, 198).

The initial step is attachment of the virus to cell receptors, mediated by the HN polypeptide. Fusion of the viral and cell membranes is brought about by action of the fusion (F) protein, and, thus, the nucleocapsid complex enters the cell.

Intracellular virus replication takes place entirely within the cytoplasm. Because the virus RNA has negative sense, the viral RNA-directed RNA-polymerase (transcriptase) must produce complementary transcripts of positive sense that may act as messenger RNA and use the cell's mechanisms, enabling the translation into proteins and virus genomes. The F protein is synthesized as a nonfunctional precursor, F0, that requires cleavage to F1 and F2 by host proteases. The significance of this cleavage in the pathogenicity of NDV strains is discussed later in this chapter. The HN of some strains of NDV may also require posttranslational cleavage.

The viral proteins synthesized in an infected cell are transported to the cell membrane, which becomes modified by their incorporation. Following the alignment of the nucleocapsid close to modified regions of the cell membrane, virus particles are budded from the cell surface.

Susceptibility to Physical and Chemical Agents

The infectivity of NDV and other avian paramyxoviruses may be destroyed by physical and chemical treatments such as heat, irradiation (including light and ultraviolet rays), oxidation processes, pH effects, and various chemical compounds. The rate at which infectivity is destroyed depends on the strain of virus, the length of time of exposure, the quantity of virus, the nature of the suspending medium, and the interactions between treatments. No single treatment can guarantee destruction of all viruses but may result in a low probability of infective virus remaining. Lancaster (157) and Beard and Hanson (38) provide detailed reviews of the early work.

Knowledge of the heat inactivation of vND is important because the virus is likely to be present in meat and other products obtained from infected poultry. The Terrestrial Animal Health Code of the World Organisation for Animal Health allows international trade in processed poultry products, even from countries with enzootic vND, but merely states that these products should "have been processed to ensure the destruction of the ND virus" (190). Alexander and Manvell (14) have produced data on the inactivation of vNDV strain Herts 33 in artificially infected chicken meat homogenate and obtained the following D_t values (i.e., the time taken to reduce the virus titer by 90 percent or 1 $\log_{10}$ at the specified temperatures): 65°C 120 secs, 70°C 82 secs, 74°C 40 secs, and 80°C 29 secs.

Egg products also represent a hazard when derived from eggs laid by infected hens. Gough (98) studied the inactivation of vNDV in liquid whole egg, and from the data published in that study, Alexander and Chettle (13) deduced that the D_t value for strain Beaudette C (a strain considered to show some heat resistance) in liquid whole egg was 38 secs at a temperature of 64.4 °C. Although not calculating D_t values, King (142) obtained data that suggested for strains Ulster and California/1083/72 very much longer survival in albumen or yolk at 57°C. In a more comprehensive study using two low virulence strains, Ulster and B1, and the vNDV strain California/02, Swayne and Beck (240) conducted a series of experiments aimed at assessing the heat inactivation of ND viruses in various egg products at temperatures

used commercially. They concluded that commercial pasteurization processes are likely to reduce any infectious ND virus present to an acceptable level, but stress that little data exists on the titers of virus likely to be present in eggs.

Strain Classification

The term *strain* is generally used to mean a well-characterized isolate of the virus. The important objective in characterizing viruses is to group similar viruses. For NDV isolates, this has inevitably meant the distinction between viruses of high and low virulence for chickens or perhaps more pertinently between enzootic and epizootic viruses.

Pathogenicity tests are useful markers and guides to the importance of the isolate. They do not indicate epizootiologic links between strains with the same virulence. Certain unrelated biologic properties of viruses have been shown to vary with different strains and isolates, and these properties have been used to characterize and group isolates.

Antigenicity

Virus neutralization (VN) or agar gel diffusion techniques have shown minor antigenic variations between different strains and isolates of NDV (97, 199, 224). For all practical purposes, however, isolates of NDV have been considered to represent a single antigenically homogeneous group.

Monoclonal antibodies (MABs) have been employed to demonstrate antigenic variation of NDV strains and isolates (2, 17, 20, 79, 124, 128, 155, 173, 186, 216, 235).

Monoclonal antibodies may detect slight variations in antigenicity, such as single amino acid changes at the epitope to which the antibody is directed. As a result, they can detect differences not only between strains but between subpopulations of virus (111). Some workers have used MABs to distinguish between specific viruses. For example, two groups have described MABs that distinguish between the common vaccine strains, Hitchner B1 and La Sota (79, 173), and other MABs can separate vaccine viruses from epizootic virus in a given area (235).

The most comprehensive use of MABs for strain characterization and classification has been by Russell and Alexander (216) and Alexander *et al.* (17, 19, 20, 21). They used MABs to place strains and isolates of NDV into groups on the basis of their ability to react with the different MABs. Viruses in the same MAB group shared biologic and epizootiologic properties. Russell *et al.* (219) pointed out the similarity between groups of viruses formed on a genetic basis and those formed on the basis of similarities in antigenicity detected using MABs.

Monoclonal antibody typing was also used to establish the uniqueness of the variant NDV responsible for the pigeon panzootic and to confirm its presence in many countries (17, 20, 196).

Pathogenicity Tests

The first attempt to distinguish between or to group isolates by a laboratory test was by assessment of virulence. Hanson and Brandly suggested that strains of NDV could be conveniently grouped as "velogenic," "mesogenic," and "lentogenic" based on chicken embryo mortality at less than 60 hr, 60–90 hr and greater than 90 hr, respectively, after allantoic inoculation (112). The values obtained provided a guide to the disease produced in infected chickens. These terms have come to be applied to high-virulence, moderate-virulence, and low-virulence viruses regardless of the method of assessment.

Other tests devised to distinguish between strains give a direct assessment of the clinical signs or deaths in infected birds. This assessment enables quantification by designating scores according to the degree of severity and calculating a pathogenicity index. The most widely used tests are the intracerebral pathogenicity index (ICPI) in day-old chicks and the intravenous pathogenicity index (IVPI) in 6-week-old chickens. The ICPI test is required by the World Organisation for Animal Health (OIE) for the *in vivo* determination of virus virulence (see below).

Genetic Characterization

Improved techniques for nucleotide sequencing, the availability of sequence data of more ND viruses placed in computer databases, and the demonstration that even relatively short sequence lengths could give meaningful results in phylogenetic analyses have essentially led to the establishment of genetic characterization of NDV strains in recent years. Particular attention has been paid to the fusion gene as this lends itself to virulence predictions. Considerable genetic diversity has been detected, but viruses sharing temporal, geographical, antigenic, or epidemiological parameters tend to fall into specific lineages or clades, and this has proven valuable in assessing both the global epidemiology and local spread of NDV (7, 24, 67, 117, 144, 145, 162, 221, 225, 226, 241, 246). Aldous *et al.* (7) proposed that genotyping of NDV isolates should become part of the diagnostic virus characterization for reference laboratories by producing a 375-nucleotide sequence of the F gene, which includes the F0 cleavage site, routinely for all viruses and comparing the sequences obtained with other recent isolates and 18 viruses representative of the recognized lineages and sublineages.

One interesting observation is that viruses with specific genetic characteristics do not necessarily disappear as other variants arise and may continue to be isolated many years after their first appearance (162).

Although less data exist than for F, the sequences of the HN gene for several strains have been determined. An HN0 precursor protein is produced for some strains (i.e., Ulster 2C (180) and D26 (222)) but not others (i.e., the lentogenic Hitchner B1 (134); mesogenic Beaudette C (179); two velogenic strains, Australia Victoria (168) and Italien (253); and the pigeon variant virus (66), which all have termination codons located before the end of the HN gene so the HN0 protein is not produced, and posttranslational cleavage is not required). In certain circumstances, this potential extension has been used to distinguish between endemic viruses of low virulence and other viruses (88).

Laboratory Host Systems

Animals

NDV can infect and multiply in a range of nonavian (156) as well as avian (136) species following laboratory infection. The chicken, however, remains the most readily available and fre-

quently used laboratory animal, as well as the most important natural host of the disease.

Chicken Embryos

All avian paramyxoviruses replicate in embryonated chicken eggs. Because of their availability (especially from specific pathogen-free sources), their sensitivity for virus growth, and the high titers to which viruses grow in them, they are generally used for virus isolation and propagation.

Newcastle disease virus strains and isolates vary in their capacity and time taken to kill chick embryos. Virus titers are also influenced by strain, with the highest titers obtainable by those causing slow or no embryo death (100). With some strains, embryo death and virus growth are affected by the presence of maternal antibodies in the yolk (86).

The route of inoculation is also important (38). Inoculation of NDV via the yolk sac, as compared with the allantoic cavity, produced more rapid embryo deaths and caused deaths by strains that do not consistently kill by the latter route (82).

Cell Cultures

Newcastle disease virus strains can replicate in an enormous range of cells. For example, Lancaster (156) listed 18 primary cell types and 11 cell lines as susceptible. Many more have been added to the list since his 1966 report. Cytopathic effects (CPE) are usually the formation of syncytia with subsequent cell death, with the CPE having some relationship to the strain's virulence for chickens (205). Plaque formation in chick embryo cells is restricted to velogenic and mesogenic viruses unless Mg^{2+} ions and DEAE (33) or trypsin (212) are added to the overlay.

Because of relatively poor growth of NDV in most cell culture systems, they are impracticable for virus propagation for most purposes.

Pathogenicity

The virulence of NDV strains varies greatly with the host. Chickens are highly susceptible, but ducks may be infected and show few or no clinical signs, even with strains lethal for chickens (118).

In chickens, the pathogenicity of ND is determined chiefly by the strain of virus, although dose, route of administration, age of the chicken, and environmental conditions all have an effect. In general, the younger the chicken, the more acute the disease. With virulent viruses in the field, young chickens may experience sudden deaths without major clinical signs; however, in older birds the disease may be more protracted and with characteristic clinical signs. Breed or genetic stock does not appear to have a significant effect on the susceptibility of chickens to the disease (64). Natural routes of infection (nasal, oral, and ocular) appear to emphasize the respiratory nature of the disease (37), and intramuscular, intravenous, and intracerebral routes appear to enhance the neurologic signs (38).

Molecular Basis for Pathogenicity

During the replication of NDV, the functionally important fusion protein is produced as a precursor glycoprotein, F0, which has to be cleaved to F_1 and F_2 for the progeny virus particles to be infectious (213). This posttranslation cleavage is mediated by host cell proteases (182). If cleavage fails to take place, noninfectious virus particles are produced. Trypsin can cleave F0 for all NDV strains, and *in vitro* treatment of noninfectious virus will restore infectivity (183).

The importance of F0 cleavage was easily demonstrated, because viruses normally unable to replicate or produce plaques in cell culture systems were able to do both if trypsin was added to the agar overlay or culture fluid. Although all viruses could replicate and produce infectious progeny in the allantoic cavity, the viruses pathogenic for chickens could replicate in a wide range of cell types *in vitro* with or without added trypsin, whereas strains of low virulence could replicate only when trypsin was added (211, 212). Thus, F0 molecules of virulent viruses can be cleaved by a host protease or proteases found in a wide range of cells and tissues, but F0 molecules in viruses of low virulence were restricted in their sensitivity to cleavage by specific host enzymes. Consequently these viruses can grow only in certain host cell types.

Early reports of the deduced amino acid sequences of the F0 precursor, obtained from nucleotide sequencing of the F gene for a number of NDV strains (59, 65, 94, 168, 178, 223, 245), enabled comparison of viruses of low virulence to those that were velogenic or mesogenic. For all viruses, the amino acid at residue 116, the C terminus of the F2 protein at the site of cleavage, was arginine. The viruses of low virulence all had leucine at residue 117, the N-terminus of the F1 protein, and another basic amino acid at residue 113. In contrast, all velogenic or mesogenic viruses had phenylalanine at residue 117 and, with one exception, basic amino acids at residues 115 and 112 in addition to those at 113 and 116. The exception was the pigeon variant PMV-1 virus, which was identical to the virulent viruses but lacked a basic amino acid at position 112. Further studies have indicated that this variation was usual for pigeon variant PMV-1 viruses but had no significance in the variability of pathogenicity for chickens recorded with these viruses (66).

Thus, it would appear that the mechanism controlling the pathogenicity of NDV is very similar to that described for influenza viruses (251). The presence of additional basic amino acids in virulent strains means that cleavage can be effected by protease or proteases present in a wide range of cell types in different host tissues and organs. This ubiquitous enzyme has not been fully identified, but as with avian influenza viruses, it is likely to be one or more proprotein-processing, subtilisin-related endoprotease[s] of which furin is the leading candidate (87, 238). For lentogenic viruses, cleavage can occur only with proteases recognizing a single arginine, i.e., trypsin-like enzymes. Lentogenic viruses, therefore, replicate only in cells where there are trypsin-like enzymes, such as the respiratory and intestinal epithelia, whereas virulent viruses can replicate in cells located in a wide range of tissues and organs, resulting in a fatal systemic infection (211).

As more sequence data have become available, a degree of variation at the F0 cleavage site has become apparent. The reported cleavage site motifs are listed in Table 3.2. During the in-

tense investigations and surveillance work following the outbreaks of ND in Australia during 1998–2000, several viruses showing variation in the amino acids at the F0 cleavage site were isolated (254). These, too, are presented in Table 3.2. The variations in these naturally occurring viruses confirm that the minimum requirement for ND viruses to show high virulence for chickens appears to be the motif ^{113}RXR/KR*F^{117}. The reason for the absolute requirement for phenylalanine at position 117 is unclear and may not be part of the recognition motif of the ubiquitous protease. Studies to determine the precise minimum amino acid motif at the F0 cleavage site to confer virulence have been undertaken using cDNA clones of NDV and reverse genetics techniques (198). De Leeuw *et al.* (76) generated a range of viruses with substituted amino acids at the F0 cleavage site (Table 3.2). They concluded that virulence required F at position 117, R at 116, K or R at 115, and R not K at 113. Interestingly, all their generated mutants reverted to the virulent motifs ^{112}RRQRR*F^{117} or ^{112}RRQKR*F^{117} after a single passage in chicks.

Although the cleavage site amino acid sequence of the F0 protein is an excellent guide to real or potential virulence of ND viruses, remember that other factors associated with other virus genes and proteins might cause variation in virulence. For example, using reverse genetic techniques, it has been demonstrated that the HN protein may influence the virulence (125, 209).

Emergence of Virulent Viruses

The greater understanding of the molecular basis for virulence has given an insight into how viruses that cause vND may emerge. Hanson (108, 109) put forward three suggestions to account for the sudden emergence of virulent NDV: 1) The virus had always been in poultry, but was unnoticed until the development of commercial poultry industries; 2) the virulent virus was enzootic in another species in which it showed less severe disease; and 3) the virulent virus arose from virus of low virulence by mutation.

Until recent years the consensus opinion has been that the second explanation was the most likely. The first was considered possible but unlikely due to the widespread outbreaks of vND now seen regularly in backyard and village chickens in some parts of the world. The third was considered unlikely because there were no reports of other viruses mutating to virulence in this way, and the degree of genetic change needed would be too large for simple mutation.

The second explanation was apparently supported by the finding during the 1970–73 panzootic that virus was introduced into some geographical areas by the movement of captive caged birds, especially psittacine species (85, 250), which show some resistance to the viruses virulent for chickens (80, 81). Although captive caged birds have often been shown to be infected with virulent NDV (192, 228), it has been suggested this may be a result of contact with infected poultry (136). Equally, apart from cormorants in North America (152) and possibly pigeons, reports of reservoirs of virulent NDV in wild birds have been lacking.

The first hint that the third explanation, that virulent viruses arise by mutation from viruses of low virulence, may account for the emergence of virulent NDV came from studies on viruses responsible for vND outbreaks in Ireland in 1990. These viruses were shown to be closely related to variant viruses of low virulence usually isolated from waterfowl, but both were antigenically and genetically distinct from all other NDVs (21, 67). The virulent viruses showed four differences in the nucleotides of the part of the F gene coding for amino acids 112 to 117, three of these resulting in a change to the assumed minimum motif for virulence, and the fourth giving a lysine at 112 (11). Much better evidence for mutation to virulence has come from the 1998–2000 vND outbreaks in Australia (146, 254). Phylogenetic studies showed the virulent viruses responsible for the outbreaks in Australia in 1998 and 1999 to be extremely closely related to each other and to a virus of low virulence isolated from chickens in the same geographical area (103). This suggested that the virulent virus emerged by mutation, which, as shown in Table 3.3, in this instance required only two point mutations. Furthermore, viruses

Table 3.2. Amino acid sequences at the F0 cleavage site of NDV strains.

Virus strain	Virulence for chickens	Cleavage site amino acids 111 to 117	Reference
Herts 33	High	-G-R-R-Q-R-R*F-	246
Essex '70	High	-G-R-R-Q-K-R*F-	65
135/93	High	-V-R-R-K-K-R*F-	187
617/83	High	-G-G-R-Q-K-R*F-	66
34/90	High	-G-K-R-Q-K-R*F-	65
Beaudette C	High	-G-R-R-Q-K-R*F-	65
La Sota	Low	-G-G-R-Q-G-R*L-	65
D26	Low	-G-G-K-Q-G-R*L-	246
MC110	Low	-G-E-R-Q-E-R*L-	65
1154/98	Low	-G-R-R-Q-G-R*L-	11
Australian isolates			
Peats Ridge	Low	-G-R-R-Q-G-R*L-	254
NSW 12/86	Low	-G-K-R-Q-G-R*L-	254
Dean Park	High	-G-R-R-Q-R-R*F-	254
Somersby 98	Low	-G-R-R-Q-R-R*L-	254
PR-32	?	-G-R-R-Q-G-R*F-	254
MP-2000	Low	-G-R-R-Q-K-R*L-	254
Generated viruses			
L (La Sota)	Low	-G-G-R-Q-G-R*L-	76
tag	High	-G-R-R-Q-R-R*F-	76
FM	Low	-G-R-R-Q-R-R*L-	76
FM1	Low	-G-G-R-Q-G-R*F-	76
FM2	Low	-G-R-R-Q-G-R*F-	76
FM3	High	-G-R-G-Q-R-R*F-	76
FM4	Low	-G-R-K-Q-K-R*F-	76
FM5	High	-G-R-R-Q-K-R*F-	76

*Represents cleavage point. Basic amino acids are shown; note that all naturally occurring virulent viruses have phenylalanine (F) at position 117, the F1 N-terminus.

Table 3.3. Nucleotide/amino acid sequence at F0 cleavage site of NDV of high and low virulence isolated in Australia in 1998.

Virus	Virulence	Nucleotide/amino acid sequence at F0 cleavage site
1154/98	Low	GGA AGG AGA CAG GGG CGT CTT ^{111}GRRQGR*L^{117}
1249/98	High	GGA AGG AGA CAG AGG CGT TTT ^{111}GRRQRR*F^{117}

From (11).

showing other changes at the cleavage site, including those intermediate to the two viruses were isolated (Table 3.2). The overwhelming probability is that the virulent ND viruses emerging in Australia in 1998 were the result of mutation from viruses of low virulence, and there is no reason to suppose that other similar mutations have not taken place in the past. Shengqing *et al.* (229) produced experimental evidence that supported the emergence of virulent ND viruses from those of low virulence. They showed that a waterfowl isolate of low virulence, with the F0 cleavage site sequence ERQER*L, passed through chickens nine times by the air sac inoculation route and then given five passages by inoculation intracerebrally, became extremely virulent for chickens with the F0 cleavage site sequence KRQKR*F.

Pathobiology and Epidemiology

Incidence and Distribution

The almost universal use of ND vaccines in commercial poultry throughout the world makes assessment of the true geographic distribution of Newcastle disease (i.e., in terms of birds infected with virulent virus) difficult. Equally, often a distinction is made between ND in commercial poultry and village and backyard chickens when reporting outbreaks. Even though international monitoring of Newcastle disease is carried out by agencies such as the Food and Agriculture Organization of the United Nations (84) and the World Organisation for Animal Health, the figures produced may not represent the true distribution of vND.

No doubt exists that vND is either enzootic or a cause of regular epizootics in poultry throughout most of Africa, Asia, Central America, and parts of South America. In more developed areas, such as Western Europe, sporadic epizootics occur on a fairly regular basis despite the widespread use of vaccination.

The distribution of vND is dependent on the attempts at eradication and control made in different countries. The success of such measures is, in turn, dependent on the nature of the poultry industry (i.e., countries with mostly village chicken flocks have far greater problems than those with mostly large commercial flocks).

The nature of the spread of vND also affects the distribution. Alexander (11) considered that probably four panzootics of vND had occurred since the first recognition of the disease. The first panzootic represented the initial outbreaks of disease and appears to have arisen in Southeast Asia. Doyle (78) considered that the disease moved slowly through Asia to Europe and that isolated outbreaks such as in England in 1926 were chance introductions ahead of the mainstream. This theory of panzootic spread of vND would mean that the virus, which had apparently arisen in 1926, took more than 30 years to spread worldwide and was still important in most countries in the early 1960s.

In marked contrast, the second panzootic appears to have begun in the Middle East in the late 1960s and to have reached most countries by 1973. The more rapid spread of the second panzootic could be because the poultry industry had undergone a major revolution in that it had developed into a major commercial industry with considerable international trade. In addition, the virus responsible for this panzootic appeared to be associated with an imported caged psittacine species. The enormous trade in these birds, which involved rapid, airborne shipments, was considered to be a major factor in the spread of the disease (85, 250).

The serious effects of the second panzootic on the poultry industries of most countries led to the development of vaccines and regimens that provided significant protection to poultry. In addition, most countries imposed new control measures for the importation of exotic caged birds. Alexander (11) considered that antigenic and genetic evidence (21, 162) indicates that there was probably worldwide spread of a third virulent virus during the late 1970s. The start and spread of this third panzootic are unclear, presumably as a result of the almost universal use of vaccines since the mid 1970s, which would have protected birds from disease but in most cases allowed replication and spread of the virus. Monitoring of viruses responsible for panzootics is further complicated, as it would appear that viruses with specific genetic or antigenic characteristics do not necessarily disappear, as other variants arise and may continue to be isolated many years after their first appearance (21, 162).

Another group of domesticated birds that was generally ignored as a potential source of vND, however, existed in large numbers in most countries. This group consisted of the pigeons and doves (*Columba livia*) that are kept for racing, show, or food purposes; in most European countries, this group may represent populations of several million birds. These were the birds primarily affected by the fourth panzootic of vND. The disease, which resembled the neurotropic form in chickens but without respiratory signs, apparently arose in the Middle East in the late 1970s (137). By 1981, it had reached Europe (45) and then spread rapidly to all parts of the world, largely as a result of contact between birds at races and shows and the large international trade in such birds. The variant nature of the virus enabled unequivocal demonstration of infection in 24 countries (17, 20, 196). Spread to chickens has occurred in several countries including Great Britain where 20 outbreaks in unvaccinated chickens occurred in 1984 as a result of feed that had been contaminated by infected pigeons (18). The disease in pigeons has been recognized for over 25 years but still seems to remain enzootic in racing pigeons in many countries, with regular spread to wild pigeons and doves and a continuing threat to poultry.

Natural and Experimental Hosts

From the available literature, Kaleta and Baldauf (136) concluded that in addition to the domestic avian species, natural or

experimental infection with NDV has been demonstrated in at least 241 species from 27 of the 50 orders of birds. These authors stressed the variation in severity of clinical signs, even with different species of a genus. Since that review, the number of species from which NDV has been isolated, with or without clinical signs, has greatly increased. It seems reasonable to conclude that the vast majority of, if not all, birds are susceptible to infection, but the disease seen with any specified strain of virus may vary considerably with host.

Transmission

In reviewing the modes of transmission of NDV between birds, Alexander (9) concluded that infection may take place by either inhalation or ingestion and that spread from one bird to another depends on the availability of the virus in an infectious form. It is tempting to assume that NDV is primarily transmitted by fine aerosols or large droplets that are inhaled by susceptible birds. Experimental evidence to prove this conclusively is, however, lacking. It is clear that infectious virus may be present in aerosols and that birds placed in an atmosphere containing such aerosols become infected. This is the basis for mass application of live vaccines by spray and aerosol generators (170). In naturally occurring infections, large and small droplets containing virus will be liberated from infected birds as a result of replication in the respiratory tract or as a result of dust and other particles, including feces. These virus-laden particles may be inhaled or impinge upon the mucous membranes, resulting in infection. The ability of such aerosols to form and to support infectious virus for a sufficient period for transmission, however, depends on many environmental factors.

During the course of infection of most birds with NDV, large amounts of virus are excreted in the feces. Ingestion of feces results in infection; this is likely to be the main method of bird-to-bird spread for avirulent enteric NDV and the pigeon variant virus (16), neither of which normally produces respiratory signs in infected birds. In experiments in which the virus was presented to the back of the buccal cavity, it was suggested that doses of vNDV as high as 10^4 EID_{50} may be required to infect 3-week-old chickens by this route, which represented perhaps as much as a gram of feces (22).

Vertical transmission (i.e., passing of virus from parent to progeny via the embryo) remains controversial. The true significance of such transmission in epizootics of ND is not clear. Experimental assessment using virulent viruses is usually hampered by cessation of egg laying in infected birds. Infected embryos have been reported during naturally occurring infections of laying hens with virulent virus (38, 157), but this generally results in the death of the infected embryo during incubation. Cracked or broken infected eggs may serve as a source of virus for newly hatched chicks, as may virus-laden feces contaminating the outside of eggs. Virus may also penetrate the shell after laying (257), further complicating the assessment of true vertical or transovarian transmission. Infected chicks may be hatched from eggs infected with vaccinal or other lentogenic viruses that do not necessarily cause death of the embryo (68, 86). In naturally occurring infections, it is not clear how the embryos become infected, although La Sota vaccine has been shown to be present in most of the reproductive organs after vaccination (204).

Pospisil *et al.* (201) were able to demonstrate the presence of lentogenic virus in chick embryos and young progeny, including day-old chicks, of a vaccinated laying flock. Capua *et al.* (58), investigating the unexpected isolation of virulent virus from chick embryos, were able to isolate virulent NDV from cloacal swabs taken from the birds that had laid the eggs, despite high antibody titers to NDV, and from their hatched progeny. In experiments, Chen and Wang (61) demonstrated that when SPF eggs were infected with very low doses of vNDV not all the embryos died, and virus could be isolated from a small number of hatched chicks.

Spread

Lancaster and Alexander (9, 156, 157) reviewed the modes of spread of NDV. The following virus sources or methods have been implicated in various epizootics: 1) movement of live birds: feral birds, pet/exotic birds, game birds, racing pigeons, and commercial poultry; 2) contact with other animals; 3) movement of people and equipment; 4) movement of poultry products; 5) airborne spread; 6) contaminated poultry feed; 7) contaminated water; and 8) vaccines.

The importance of any of these factors will depend on the situation in which the epizootic occurs. In countries where poultry are kept exclusively in birdproof housing, the ability of feral birds to invade affected flocks and transfer the disease will be minimal, whereas birds kept on open range are more likely to be infected with strains carried by feral birds. In Canada and the northern United States, outbreaks of vND occurred in double-crested cormorants and pelicans in 1990 and 1992 (31, 247, 262), and sporadic outbreaks in cormorants have continued since that time. A virus, indistinguishable from the cormorant viruses using MAB panels and essentially the same genetically, was also isolated from turkeys showing signs of neurotropic velogenic ND that had been kept on range in North Dakota in the vicinity of diseased cormorants from which vNDV had been isolated (225, 247). Further studies resulted in the isolation of vND virus from cormorants on their wintering grounds in Florida, in 2002 (28). Nucleotide sequencing of a portion of the F gene of this virus showed 100% deduced amino acid for that part of the F protein with the 1992 North U.S. cormorant virus and the isolate from turkeys.

It was also thought that the 11 outbreaks confirmed in chickens and turkeys in Great Britain between early January and late April 1997 (23) may have been the result of primary introduction of the causative virus by migratory birds. Epidemiological investigations suggested that most of the outbreaks were the result of secondary spread by humans from one or two primary introductions. However, nucleotide sequencing and phylogenetic analysis showed very close similarity between the British isolates and the viruses responsible for vND outbreaks in Scandinavian countries in 1996, including an isolate from a feral goosander (24). The unusual patterns of movement of migratory birds at the end of 1996 and the beginning of 1997 suggest that they may have been the vehicle for the primary introduction of the vND virus into Great Britain.

In spite of continuing international trade in exotic caged birds and the frequent isolation of virulent NDV from such birds (228), the threat of introduction and spread by this source (as in the California epizootic in 1971–1972) (248) has been greatly reduced by strict importation quarantine procedures. However, smuggled birds or those removed prematurely from quarantine may still pose a threat (228); since 1973, virulent NDV has been isolated regularly from pet birds imported illegally or held in quarantine in the United States (192). There was particular concern in 1991 when outbreaks occurred in illegally imported pet birds in six states (56, 192); however, there was no spread to poultry.

Backyard game fowl (fighting cocks) have been involved in outbreaks of vND in the United States on three occasions, 1975, 1998, and 2002–2003 (32, 140, 147). The most notable outbreak occurred in southern California in 2002–2003 (140) in which more than 149,000 birds in 2,671 premises were destroyed to control the disease. The mobility and value of such birds increase the risk of spreading ND during outbreaks of disease because owners seek to avoid quarantine or possible destruction of the birds by regulatory authorities. It is suspected that the spread of vND from southern California to adjacent states of Nevada and Arizona was due to the movement of infected birds or contaminated materials. Although initially the disease was present primarily in backyard game fowl, the disease spread to 21 commercial table–egg layer flocks (3 million birds) in southern California. The highest risk factors for infected commercial flocks were the farm employees and proximity to infected backyard game fowl.

Airborne spread has been considered to be important in some epizootics such as the 1970–1971 outbreaks in England (127) but unimportant in others such as the 1971–1972 California outbreaks (248), even though the same virus appears to have been involved. In more recent outbreaks, airborne spread has generally not been considered to contribute to any spread that has occurred.

In some cases, more than one factor combines in the spread of the disease. For example, the 1984 outbreaks of vND in Great Britain were considered to be spread by feed that had been contaminated by infected feral pigeons (18).

Without doubt, the greatest potential for spread of NDV is by humans and their equipment. Humans may be infected in the conjunctival sac with NDV, and this could pose a method of spread, but a more probable method is the mechanical transfer of infective material (most probably feces). Modern transportation enables personnel to travel rapidly to any country in the world, so spread by humans should not be treated as merely a local or national threat.

Vaccination crews moving from farm to farm have been implicated in the spread of NDV (248), as have incomplete inactivation (233) and contamination of vaccines (40, 135).

Incubation Period

The incubation period of ND after natural exposure has been reported to vary from 2–15 days (average 5–6). The speed with which signs appear, if at all, is variable depending on the infecting virus, the host species and its age and immune status, infection with other organisms, environmental conditions, the route of exposure, and the dose.

Clinical Signs

Newcastle disease virus isolates can be broadly grouped into pathotypes on the basis of clinical signs, which in turn are affected by the strain of virus. Other factors also important in establishing the severity of the disease are the host species, host age, host immune status, coinfection with other organisms, environmental stress, social stress, route of exposure, and the virus dose (166).

With extremely virulent viruses, the disease may appear suddenly, with high mortality occurring in the absence of other clinical signs. In outbreaks in chickens due to the VVND pathotype, clinical signs often begin with listlessness, increased respiration, and weakness, ending with prostration and death. During the panzootic caused by this type of virus in 1970–1973, disease in some countries such as Great Britain (27) and Northern Ireland (166) was marked by severe respiratory signs, but in other countries these were absent. This type of vND may cause edema around the eyes and head. Green diarrhea is frequently seen in birds that do not die early in infection, and prior to death, muscular tremors, torticollis, paralysis of legs and wings, and opisthotonos may be apparent. Mortality frequently reaches 100% in flocks of fully susceptible chickens.

The neurotropic velogenic form of ND has been reported mainly in the United States. In chickens, it is marked by sudden onset of severe respiratory disease followed a day or two later by neurologic signs. Egg production falls dramatically, but diarrhea is usually absent. Morbidity may reach 100%. Mortality is generally considerably lower, although up to 50% in adult birds and 90% in young chickens have been recorded.

Mesogenic strains of NDV usually cause respiratory disease in field infections. In adult birds, there may be a marked drop in egg production that may last for several weeks. Nervous signs may occur but are not common. Mortality in fowl is usually low, except in very young and susceptible birds, but may be considerably affected by exacerbating conditions.

Lentogenic viruses do not usually cause disease in adults. In young, fully susceptible birds, serious respiratory disease problems can be seen, often resulting in mortality, following infection with the more pathogenic La Sota strains complicated by infections with one or more of a range of other micro-organisms. Vaccination or infection of broilers close to slaughter with these viruses can lead to colisepticemia or airsacculitis, with resulting condemnation.

The virus responsible for the panzootic in pigeons during the 1980s induced clinical signs in field infections of pigeons (249) and chickens (18) unlike those from other viruses. In both species, the predominant clinical features were diarrhea and nervous signs. In adult chickens, precipitous falls in egg production were seen, and high mortality was recorded in younger birds. This virus did not induce respiratory signs in uncomplicated infections of pigeons or chickens.

The clinical signs produced by specific viruses in other hosts may differ widely from those seen in chickens. In general, turkeys

are as susceptible as chickens to infection with NDV, but clinical signs are usually less severe (25, 50, 166). Although readily infected, ducks and possibly geese usually are regarded as clinically resistant even to the strains of NDV most virulent for chickens. However, outbreaks of severe disease in ducks infected with NDV have been described (118). Outbreaks of vND have been reported in most game bird species (156, 157), and the disease appears similar to that in chickens (43). In ostriches and other ratites, ND viruses virulent for chickens do not produce such pathogenic disease. Generally young ostrich chicks may show depression and nervous signs, but adults appear unaffected (10).

Pathology

Gross Lesions

As with clinical signs, the gross lesions and the organs affected in birds infected with NDV are dependent on the strain and pathotype of the infecting virus, in addition to the host and all the other factors that may affect the severity of the disease. No pathognomonic lesions are associated with any form of the disease. Gross lesions may also be absent.

Nevertheless, the presence of hemorrhagic lesions in the intestine of infected chickens has been used to distinguish VVND viruses from NVND viruses. (110, 113). These lesions are often particularly prominent in the mucosa of the proventriculus, ceca, and small and large intestine. They are markedly hemorrhagic and appear to result from necrosis of the intestinal wall or lymphoid tissues such as cecal tonsils and Peyer's patches.

Generally, gross lesions are not observed in the central nervous system of birds infected with NDV, regardless of the pathotype (166).

Gross pathologic changes are not always present in the respiratory tract, but when observed they consist predominantly of mucosal hemorrhage and marked congestion of the trachea (12). Airsacculitis may be present even after infection with relatively mild strains, and thickening of the air sacs with catarrhal or caseous exudates is often observed in association with secondary bacterial infections (38).

Gross pathology seen in other organs includes hemorrhage in the lower conjunctiva, focal necrosis of the spleen, and paratracheal edema, most generally observed near the thoracic inlet.

Chickens and turkeys infected in lay with velogenic viruses usually have egg yolk in the abdominal cavity. The ovarian follicles are often flaccid and degenerative. Hemorrhage and discoloration of the other reproductive organs may occur.

Gross lesions of velogenic viscerotropic Newcastle disease in susceptible chickens inoculated by the eye drop route are illustrated in Figure 3.2.

Microscopic Lesions

The histopathology of NDV infections is as varied as the clinical signs and gross lesions and can be affected greatly by the same parameters. In addition to the strain of virus and host, the route or method of infection may also be of paramount importance. For example, Beard and Easterday (37) were able to demonstrate similar histopathologic changes in the tracheas of chickens infected with either lentogenic or velogenic viruses by aerosol. Most published descriptions of the histologic changes following NDV infections are related to the virulent pathotypes, and several descriptive reports or reviews of the literature have covered the histologic changes in the various organs during infection (37, 38, 166, 255). Briefly, the major changes are as follows.

Nervous System. Lesions seen in the central nervous system are those of a nonpurulent encephalomyelitis with neuronal degeneration, foci of glial cells, perivascular infiltration of lymphocytes, and hypertrophy of endothelial cells. Lesions usually are seen in the cerebellum, medulla, midbrain, brain stem, and spinal cord, but rarely in the cerebrum.

Vascular System. Congestion, edema, and hemorrhage are found associated with the blood vessels of many organs. Other changes that may be seen consist of hydropic degeneration of the media, hyalinization of capillaries and arterioles, development of hyaline thrombosis in small vessels, and necrosis of endothelial cells of the vessels.

Lymphoid System. Regressive changes found in the lymphopoietic system consist of the disappearance of lymphoid tissue. Hyperplasia of the mononuclear phagocytic cells in various organs, especially the liver, may take place in subacute infections. Necrotic lesions are found throughout the spleen. Focal vacuolation and destruction of lymphocytes may be seen in the cortical areas and germinal centers of the spleen and thymus. Marked degeneration of lymphocytes in the medullary region is seen in the bursa of Fabricus (237).

Intestinal Tract. Hemorrhage and necrosis of mucosal lymphoid tissue are seen in the intestinal tract with infections of some virulent forms of ND. Other lesions are related to changes in the vascular system.

Respiratory Tract. The effect of NDV infection on membranes of the upper respiratory tract may be severe and related to the degree of respiratory distress. Lesions may extend throughout the length of the trachea. Cilia may be lost within two days of infection. In the mucosa of the upper respiratory tract, congestion, edema, and dense cellular infiltration of lymphocytes and macrophages may be seen, particularly following aerosol exposure (37). The process appears to clear rapidly, and birds examined as early as six days after infection may be free from inflammation.

Cheville *et al.* (62) infected birds with two U.S. viscerotropic isolates, Texas 219 and Florida Largo. Marked lesions of the lung were seen with both viruses, the former producing congestion and edema of the parabronchi and the latter more extensive lesions consisting of hemorrhage and erythrophagocytosis in the alveolar areas of the parabronchi.

Edema, cell infiltration, and increased thickness and density of the air sacs may occur in chickens.

Reproductive System. Histopathologic changes in the reproductive tract are extremely variable. Biswal and Morrill (46) reported that the greatest functional damage was to the uterus or shell-

forming portion of the oviduct. Changes in female reproductive organs included atresia of follicles with infiltration of inflammatory cells and the formation of lymphoid aggregates. Similar aggregates were present in the oviduct.

Other Organs. Small focal areas of necrosis are seen in the liver and, sometimes with hemorrhage, in the gallbladder and heart. Lymphocyte infiltration has been reported in the pancreas. In infections with the viscerotropic velogenic viruses, hemorrhage and ulceration of the skin may occur, and congestion and petechiae of the combs and wattle are common. Conjunctival lesions may be associated with hemorrhage.

Immunity

Active Immunity

Cell-Mediated Immunity. The initial immune response to infection with NDV is cell mediated and may be detectable as early as 2–3 days after infection with live vaccine strains (91, 244). This has been thought to explain the early protection against challenge that has been recorded in vaccinated birds before a measurable antibody response is seen (26, 99). However, a later study (207) concluded that the cell-mediated immune response to NDV by itself is not protective against challenge with virulent NDV. The importance of cell-mediated immunity in protection conferred by vaccines is, therefore, not clear, and a strong secondary response to challenge similar to the antibody response does not seem to occur (244).

Humoral Immunity. Antibodies capable of protecting the host can be measured in VN tests. Because the VN response appears to parallel the HI response, however, the latter test is frequently used to assess protective response, especially after vaccination (27). Antibodies directed against either of the functional surface glycopolypeptides, the HN and the F polypeptides, can neutralize NDVs (214). In fact, MABs specific for epitopes on the F polypeptide have been shown to induce greater neutralization than those directed against HN in *in vitro* and *in vivo* tests (178, 174). Therefore, the successful reliance on the simple HI test to assess protection up until now may have been fortuitous.

When chickens survive NDV infection long enough, antibodies usually are detectable in the serum within 6–10 days. The levels largely depend on the infecting strain, but generally, peak response is at about 3–4 weeks. Decline in antibody titer varies with the titer achieved but is much slower than their development. Hemagglutination inhibition antibodies may remain detectable for up to one year in birds recovered from infection with mesogenic viruses or after a series of immunizations. Reinfection or immunization some weeks after the titer begins to decline produces a secondary response (27).

Local Immunity. Antibodies appear in secretions of the upper respiratory tract and intestinal tract of chickens at about the time humoral antibodies can be first detected. In the upper respiratory tract, the immunoglobulins appear to be chiefly IgA with some IgG (194). Similar excretions occur in the Harderian gland following ocular, but not parenteral, infection (194, 202). Malkinson and Small (164) demonstrated effective local immunity when they found that birds may be susceptible to infection at one site but protected at another. The exact function of local immunity in protection is not clear, although a role in protection of the respiratory tract independent of humoral immunity has been proposed (123). Eye-drop vaccination with Hitchner B1 resulted in replication of virus in the Harderian gland, which could be prevented by the presence of maternal IgG in lachrymal fluid (215). Replication of virus in the Harderian gland resulted in the production of lachrymal IgG, IgA, and IgM (215). In particular, the Harderian gland became the main site for IgA-antibody-forming cells in the chicken (218). Russell and Ezeifeka (217) stressed that IgM may be the class of antibody most actively involved in the clearance of virus in conjunctival infections.

Passive Immunity. Hens with antibodies to NDV will pass these on to their progeny via the egg yolk (116). Levels of antibody in day-old chicks will be directly related to titers in the parent. Allan *et al.* (27) estimated that each twofold decay in maternally derived HI titer takes about 4.5 days. Maternal immunity is protective and, thus, must be taken into account when timing the primary vaccination of chicks.

Immunosuppression. Suppression of the immune response has important effects on both the pathogenicity of infecting NDV strains and the protection levels achieved by vaccination. Under natural conditions, immunosuppression may occur due to infection with other viruses such as infectious bursal disease virus. The subsequent immunodeficiency may result in a more severe disease caused by some NDV strains and a failure to respond adequately to vaccination (83, 93, 195, 210). Immunosuppression from chicken infectious anemia virus also has been implicated in the failure of chickens to respond well to secondary inactivated NDV vaccine (53).

Diagnosis

The objectives in the diagnosis of NDV infections are to reach a decision on whether or not to impose control measures and to obtain evidence to support epidemiological investigations. None of the clinical signs or lesions of vND may be regarded as pathognomonic, and the wide variation in disease with virus strain, host species, and other factors means that at best, these can serve as only a suggestion of infection with NDV. Similarly, the presence of lentogenic NDV strains in birds in most countries and the almost universal use of live vaccines means that mere demonstration of infection, without definition of the infecting virus, is rarely adequate cause for control measures to be imposed. Additionally, vND may cause such devastating epizootics and can have such far-reaching effects on trade in poultry products that control measures usually are defined at a national or international level.

Isolation and Identification of Causative Agent

Direct Detection of Viral Antigens

Immunohistologic techniques offer a rapid method for the specific demonstration of the presence of virus or viral antigens in

organs or tissues. Immunofluorescence techniques for thin sections of trachea (120) or impression smears (169) and an immunoperoxidase technique for thin sections (107, 161) have been described and used in NDV infections.

Virus Isolation of NDV

Although molecular techniques, especially those developed to employ RT-PCR directly on samples from affected birds (95), mean that a positive diagnosis at least can be obtained rapidly without virus isolation, it is still important that, for primary outbreaks especially, the virus is isolated for proper characterization and future work.

Culture System. Virulent ND viruses can be propagated in many cell culture systems, and viruses of low virulence can be induced to replicate in some of them. It is possible to use primary cell cultures or even cell lines for routine isolation of NDV. The embryonated chicken egg, however, represents an extremely sensitive and convenient vehicle for the propagation of NDV and is used almost universally in diagnosis.

Embryonated chicken eggs should be obtained from a specific-pathogen-free (SPF) flock and incubated for 9–10 days at 37°C before use. If SPF eggs cannot be obtained, eggs from a flock free of NDV antibodies should be used. NDV strains in eggs containing yolk antibodies can be propagated, but the virus titer is usually greatly reduced, and such eggs should be avoided for diagnostic use.

Samples. The two main sites of replication of NDV in infected poultry appear to be the respiratory and intestinal tracts, so specimens taken should always include either feces, intestinal contents or cloacal swabs, and tracheal swabs or tracheal samples depending on the circumstances. Other specimens taken from carcasses should reflect the clinical signs prior to death and organs obviously affected.

Newcastle disease virus is relatively stable for long periods in nonputrefying tissues provided that the ambient temperature is low (156). Gough *et al.* (102), however, considered the transport of samples in a frozen or chilled state as very important in virus isolation. Omojola and Hanson (191) suggested that bone marrow may be a useful sample in countries where transport is slow, temperatures are high, and refrigeration is not available, as they were able to isolate virus from this site after several days at 30°C.

Isolation Method. Ideally, each sample should be treated separately. It is common, however, to pool organ and tissue samples, although tracheal and fecal samples are best kept separate. Antibiotic media is used to make 20% weight/volume suspensions of feces or finely minced tissues and organs. Swabs are placed in sufficient antibiotic medium to ensure full immersion.

The suspensions are held at room temperature for 1–2 hours and then centrifuged at 1000 g for 10 minutes. Each of five embryonated eggs should then be inoculated with 0.2 mL of the supernatant fluid into the allantoic cavity. The eggs are placed at 37°C and examined regularly.

Eggs dead or dying, or after a minimum of four days and a maximum of seven days, should be chilled to 4°C, and the allantoic/amniotic fluid should be harvested. The presence of virus can be detected by an HA test; nonhemagglutinating fluids should be passaged at least one more time. Hemagglutination may be caused by bacteria, and possible contamination should be assessed by culture. If bacteria are present, the contaminated fluids can be passed through a 450-nm membrane filter before repassaging in eggs.

Table 3.4. Examples of pathogenicity indices obtained for strains of Newcastle disease virus.

Virus strain	Pathotype	ICPI[a]	IVPI[b]	MDT[c]
Ulster 2C	Asymptomatic enteric	0.0	0.0	>150
Queensland V4	Asymptomatic enteric	0.0	0.0	>150
Hitchner B1	Lentogenic	0.2	0.0	120
F	Lentogenic	0.25	0.0	119
La Sota	Lentogenic	0.4	0.0	103
H	Mesogenic	1.2	0.0	48
Mukteswar	Mesogenic	1.4	0.0	46
Roakin	Mesogenic	1.45	0.0	68
Beaudette C	Mesogenic	1.6	1.45	62
Texas GB	Velogenic	1.75	2.7	55
NY parrot 70181	Velogenic	1.8	2.6	51
Italien	Velogenic	1.85	2.8	50
Milano	Velogenic	1.9	2.8	50
Herts 33/56	Velogenic	2.0	2.7	48

Data from (12, 27).
[a]ICPI, intracerebral pathogenicity index in day-old chicks.
[b]IVPI, intravenous pathogenicity index in six-week-old chickens.
[c]MDT, mean death time (hr) for chick embryos infected with one minimum lethal dose.

Virus Characterization

The widespread presence of lentogenic strains in feral birds and the use of such viruses as live vaccines mean that isolation of NDV is rarely sufficient to confirm a diagnosis of disease. For such confirmation and to meet statutory requirements that may be in force, further virus characterization such as pathogenicity testing or nucleotide sequencing is necessary.

Pathogenicity Tests. The importance and impact of an NDV isolate will be directly related to the virulence of that isolate. Because field disease may be an unreliable measure of the true virulence of the virus, it is necessary to carry out laboratory assessment of the pathogenicity of the virus. In the past, three *in vivo* tests have been used for this purpose: 1) mean death time (MDT) in eggs, 2) ICPI, and 3) IVPI.

Examples of the values obtained in these three methods are shown for some well-characterized NDV strains in Table 3.4; it should be noted that the *in vivo* test prescribed by the World Organisation for Animal Health (OIE) is the ICPI test.

Although these pathogenicity tests have proved invaluable in distinguishing among vaccine, enzootic, and epizootic viruses

during outbreaks, some drawbacks to the tests and difficulties exist in the interpretation of results. For example, Pearson *et al.* (196) reported 10 NDV isolates from pigeons to have ICPI values between 1.2 and 1.45 and a range of IVPI values of 0 to 1.3, suggesting that the viruses be at least mesogenic; however, the lowest MDT recorded was 98 hour, a characteristic of lentogenic viruses. Meulemans *et al.* (176) characterized 27 PPMV-1 isolates from pigeons during 1998 and 1999. They showed that while these viruses all possessed F0 cleavage site motifs indicating vNDV and were antigenically indistinguishable from virus isolated in 1983–1984, the ICPI values for the 1998–1999 viruses gave a mean of 0.69 [range 0.21–1.27] compared to a mean value of 1.44 for the 1983–1984 viruses. In addition, work by Alexander and Parsons (15) on NDV (APMV-1) isolates from pigeons showed that both ICPI and IVPI values increased after passage through chickens or embryonated chicken eggs. This suggests that isolates adapted to birds other than poultry may not show their potential virulence for chickens in conventional pathogenicity tests.

In Vitro Tests for Pathogenicity. Only NDVs possessing additional basic amino acids at the cleavage site of the fusion protein are rendered infectious by nontrypsinlike proteases. Rott, therefore, suggested (212) that the ability of NDV isolates to form plaques in cell culture systems in the absence of trypsin represents a simple *in vitro* method for the detection of virulent viruses. As discussed previously, a much greater understanding of the molecular basis for pathogenicity has been obtained in recent years, and international definitions now allow for the detection of multiple basic amino acids at the F0 cleavage site to be used in place of *in vivo* tests for the confirmation of virulent ND virus (189). The use of molecular techniques in the diagnosis of ND, especially assessing the virulence of the infecting virus, is discussed later in this chapter.

Virus Property Profiles. Newcastle disease virus isolates show a marked variation in biologic and biochemical properties (see Introduction), and some workers used these properties to develop distinct profiles enabling the grouping of viruses for the purposes of diagnosis (38, 110). Under specific circumstances, single properties of the virus may be sufficient to distinguish between avirulent and virulent isolates and be usefully employed in diagnosis.

Monoclonal Antibodies. In addition to their use in routine diagnosis, panels of MABs can be used to characterize and group isolates by establishing profiles. Such typing on an antigenic basis represents a powerful tool for the diagnostician and epizootiologist, allowing rapid grouping and differentiation of NDV isolates (20, 214).

Serology

The presence of specific antibodies to NDV in the serum of a bird gives little information on the infecting strain of NDV and, therefore, has limited diagnostic value.

Nevertheless, in certain circumstances the demonstration that infection has taken place is sufficient for the needs of the diagnostician. Postvaccinal serology can be used to confirm successful application of vaccine and an adequate immune response by the bird.

Serologic Tests for Newcastle Disease Virus Antibodies

Antibodies to NDV may be detected in poultry sera by a variety of tests including single radial immunodiffusion (63), single radial hemolysis (114), agar gel precipitin (89), VN in chick embryos (36), and plaque neutralization (38). Enzyme-linked immunosorbent assays (ELISAs), which lend themselves to semiautomated techniques, have become popular, especially as part of flock screening procedures (232), and a variety of such tests have been described (4, 171, 176, 208, 231, 259). Good correlation has been reported between ELISA and HI tests (4, 54, 74). ELISA tests should be modified and validated for different host species; this problem can be overcome by use of competitive or blocking ELISAs employing one or more mAb to NDV.

Conventionally, antibodies to NDV and the other avian paramyxoviruses have been detected and quantitated by the HI test. Many methods for HA and HI tests have been described. Sera from other species (including turkeys) may cause low-titer, nonspecific agglutination of chicken RBCs, complicating the test. Such agglutination may be removed by adsorption with chicken RBCs before testing.

Although the HA and HI tests are not greatly affected by minor changes in the methodology, Brugh *et al.* (55) stressed the critical nature of the antigen/antiserum incubation period in test standardization and surveys have not always reported good reproducibility in HI tests among different laboratories (39). Variation in testing procedures, especially the antigen used, may account for the lack of reproducibility and laboratories should follow carefully procedures recommended by international agencies such as the World Organisation for Animal Health (189).

Differential Diagnosis

Viral HA activity may be due to any of the nine avian paramyxovirus serotypes or any of the 16 influenza type A hemagglutinin subtypes that are known to infect birds. Demonstration that the virus is of a specific serotype usually can be carried out by a simple HI test with specific polyclonal antisera.

Newcastle disease virus (APMV-1) shows some cross-reaction in HI tests with several of the other avian paramyxovirus serotypes, especially APMV-3 psittacine isolates, using polyclonal antisera (8). Although the potential for misdiagnosis largely can be eliminated by the use of control sera and antigens in conventional tests, the use of MABs in routine diagnosis can give an unequivocal result.

Molecular Techniques in the Diagnosis of ND

The conventional diagnostic techniques described previously cover detection and limited virus characterization but go only a little way toward supporting epidemiological investigations. In addition, these techniques are considered by many as slow and laborious, requiring unacceptable use of animals, and, above all, they are expensive. The development of molecular biological di-

agnostic techniques in recent years, added to our increasing knowledge of the molecular basis of the pathogenesis of vND, has led many workers to investigate the possibility that conventional diagnosis could be replaced by molecular-based techniques. An added attraction is that all three aspects of diagnosis possibly could be achieved in a single test. The various molecular approaches and techniques applied to ND diagnosis have been reviewed in detail by Aldous and Alexander (5). Most molecular techniques involve a polymerase chain reaction (PCR) and, because NDV has an RNA genome, reverse transcription (RT) to produce a DNA copy of the genome, which is an essential initial step.

Amplification of the DNA copy by PCR can be accomplished by using universal primers that merely identify NDV or its presence (95, 132); primers that amplify areas of the genome of viruses with specific properties, e.g., pathotype (138); or amplification that combines these, usually involving nested PCR (133, 138, 141, 149).

The generated PCR product may be designed to be specifically used in further molecular studies aimed at giving greater information on the properties or origins of the infecting virus. Such studies have included restriction enzyme analysis (30, 149, 184, 252), probe hybridization (6, 130, 131, 187, 203), and nucleotide sequencing for cleavage site analysis and epidemiological studies (24, 65, 66, 67, 72, 115, 117, 144, 145, 162, 165, 225, 226, 236, 241, 245, 246, 263).

Ideally, molecular techniques would allow the amplification of the virus genome directly from infected tissues to avoid the need to isolate the virus. However, this is hindered by the presence of PCR inhibitors in many organs and tissues, especially blood and feces (256). Nevertheless, Gohm *et al.* (95) pursued this strategy and reported success in amplifying a 182 bp product, including the cleavage site, directly from tissues of infected chickens. The main drawback was that no one tissue was always positive during a time course study, necessitating the need to sample a wide range of tissues and organs.

Several groups have reported the use of probes in a variety of tests that recognize specific sites on the NDV genome and, therefore, characterize the virus, at least identifying its virulence (6, 130, 187). The advantages of such techniques are that they are rapid, can be automated, and allow a large number of samples to be screened. The disadvantage is, similar to primary primers aimed at identifying the pathotype, that because ND viruses show considerable variation around the important cleavage site region, the designed probes are unlikely to recognize all ND viruses.

More recently, Wise *et al.* described a one-step real-time RT-PCR (rRT-PCR) assay that was shown to be highly sensitive in the detection of NDV-specific RNA in clinical samples (261). Using separate reactions, the assay can identify virulent (including many of the PPMV-1 viruses) and low virulent virus as well as mixtures of both pathotypes, which is an important feature in outbreaks when live vaccines may be used in control programs. The assay was developed and validated during an outbreak of virulent ND in the United States in 2002–2003 (140) and eventually replaced virus isolation as the primary diagnostic test for the control program. The assay is now authorized for use in 48 laboratories comprising the National Animal Health Laboratory Network (NAHLN) as a front-line surveillance tool for NDV in the United States.

Real-time RT-PCR assays have the advantage over conventional RT-PCR assays in that fluorogenic hydrolysis (Taqman) probes or fluorescent dyes are used to monitor the presence of target DNA after each PCR cycle, thus providing results in real time. In addition to speed, the primary advantage of the one-step PCR assays is the elimination of the post-amplification processing step, thus reducing laboratory contamination of samples. However, many smaller laboratories are at a disadvantage in using this technology because of the high start-up costs associated with purchasing the real-time thermocyclers.

Intervention Strategies

Regardless of whether ND control is applied at the international, national, or farm levels, the objective is either to prevent susceptible birds from becoming infected or to reduce the number of susceptible birds by vaccination. For the former strategy, each method of disease spread must be considered in prevention policies.

Management Procedures

International Control Policies

The raising of poultry and trade in their products are now organized on an international basis, frequently under the management of multinational companies. There is a desire to trade both poultry products and genetic stock. The threat of vND, however, has proved to be a great restraint on such trade. Bennejean (44) considered that worldwide control of vND will be approached only if all countries report outbreaks within their borders to international agencies. International agreements on these and other points are not simple, owing to the enormous variation in the extent of disease surveillance and diagnostic capabilities in different countries.

A prerequisite to formulating control policies, particularly internationally, would be agreement on what constitutes disease and to what viruses control policies should apply. Some countries do not vaccinate and would not want any form of NDV introduced to their domestic poultry. Others allow only specific live vaccines and consider some vaccines to be unacceptably virulent. Yet other countries have the continued presence of circulating highly virulent virus, which is not seen as overt disease because of vaccination. The World Organisation for Animal Health (OIE) is responsible to the World Trade Organization for the standardization of matters relating to animal health that would affect trade. The definition of vND adopted by OIE (189) reflects the current understanding of the molecular basis for virulence:

> *"Newcastle disease is defined as an infection of birds caused by a virus of avian paramyxovirus serotype 1 (APMV-1) that meets one of the following criteria for virulence:*
>
> *a) The virus has an intracerebral pathogenicity index (ICPI) in day-old chicks (Gallus gallus) of 0.7 or greater.*
>
> *or*

b) Multiple basic amino acids have been demonstrated in the virus (either directly or by deduction) at the C-terminus of the F2 protein and phenylalanine at residue 117, which is the N-terminus of the F1 protein. The term 'multiple basic amino acids' refers to at least three arginine or lysine residues between residues 113 to 116. Failure to demonstrate the characteristic pattern of amino acid residues as described above would require characterisation of the isolated virus by an ICPI test.

In this definition, amino acid residues are numbered from the N-terminus of the amino acid sequence deduced from the nucleotide sequence of the F0 gene, 113–116 corresponds to residues 24 to 21 from the cleavage site."

National Control Policies

At the national level, control policies are directed at prevention of the introduction of virus and prevention of virus spread within the country. To prevent the introduction of NDV, most countries have restrictions on trade in poultry products, eggs, and live poultry; these vary greatly within the parameters allowed by OIE animal health codes.

Because of the link between exotic caged birds and the spread of NDV during the 1970–1974 panzootic (85, 250) and the known ability of psittacine birds to excrete NDV for many weeks after infection (80, 81), most importing countries have established quarantine procedures for importations.

The continuing panzootic of vND (APMV-1) in racing pigeons that began in the 1980s (249) produced a unique situation, in view of the potential spread to poultry (250). Due to the large number of international pigeon races that take place each year, national policies were created in some countries including the banning of races, restricting races, or enforcing vaccination of participating pigeons.

In many countries legislation exists to control vND outbreaks that may occur. Some countries have adopted eradication policies with compulsory slaughter of infected birds, their contacts, and products. Such policies usually include restrictions of movement or marketing of birds within a defined quarantine area around the outbreak. Others require prophylactic vaccination of birds even in the absence of outbreaks, and some have a policy of "ring vaccination" around outbreaks to establish a buffer zone.

Higgins and Shortridge (119) stressed the importance of tailoring control policies to the country and warned against the dogmatic application of policies successful in one country to another that may differ socially, economically, and climatically.

Control and Prevention at the Farm Level

Possibly the most important factors in preventing the introduction of NDV and its spread during outbreaks are the conditions under which the birds are reared and the degree of biosecurity practiced at the farm. Chapter 1, "Principles of Disease Prevention: Diagnosis and Control," provides a comprehensive discussion of disease prevention through sanitation and security practices.

Although many biosecurity measures may often be regarded as costly, onerous, and time-consuming by those involved, if such measures are implemented there is no doubt that the introduction of ND viruses to poultry flocks and the spread to the rest of the poultry industry will be dramatically reduced. Such measures are also likely to reduce the spread of other endemic diseases that may affect the birds and reduce their yield and should be seen as an important investment in the profitability of poultry production.

Vaccination

Ideally, vaccination against vND would result in immunity against infection and replication of the virus. Realistically, ND vaccination usually protects the bird from the more serious consequences of disease, but virus replication and shedding may still occur, albeit at a reduced level (25, 104, 193, 248).

Allan *et al.* (27) produced a comprehensive description of all aspects of ND vaccination and vaccine production. Other detailed reviews have been published by Meulemans (170) on the use of vaccination in the control of ND, by Cross (73) on vaccine production, and by Thornton (243) on quality control of vaccines.

It should be emphasized that in no circumstances can vaccination be regarded as an alternative to good management practice, biosecurity, or good hygiene in rearing domestic poultry.

Historical Aspects of Vaccination

Early studies demonstrated that inactivated infective material conferred protection on inoculated chickens, but problems in production and standardization discouraged its use on a large scale. Studies in the 1930s on the attenuation of virulent NDV strains by Iyer and Dobson led to the development of mesogenic vaccine strains that are still in use in some parts of the world (105, 129).

The identification of ND in the United States (35) led initially to the use of inactivated vaccines (122). The later observation that some of the enzootic viruses produced only mild disease resulted in the development of the mesogenic live vaccine Roakin (42) and, subsequently, the milder Hitchner B1 (121) and La Sota (96) strains, which are now the most widely used vaccines.

Inactivated vaccines, usually with the virus adsorbed to aluminum hydroxide, were most widely used in Europe up to the 1970–1974 panzootic, but their poor performance at that time resulted in the adoption of live vaccination with B1 and La Sota in most countries. This panzootic also supplied the impetus for the development of modern inactivated vaccines based on oil emulsions, which have proven highly effective.

Vaccination Policies

Some governments have legislation affecting the use and quality control of vaccines. Policies vary enormously, in line with the enzootic status or perceived threat of ND. Some countries, such as Sweden, ban the use of any vaccine, and others (e.g., The Netherlands) enforce vaccination of all poultry. Countries of the European Union have legislated to define the pathogenicity of viruses that will be allowed for use as vaccines in member states. The master seed of live vaccines must be tested under specified dose conditions and shown to have an ICPI value of less than 0.4, and the master seed of viruses used in inactivated vaccines must have an ICPI value less than 0.7 (71). Similar guidelines were adopted by OIE (188).

Table 3.5. Examples of Newcastle disease viruses used as live vaccines.

Virus	Pathotype	ICPI[a]	Derivation	Use in chickens	Routes[b]
La Sota	Lentogenic	0.4	Field isolate	Primary	in, io, dw, sp, aer[c]
F (Asplin)	Lentogenic	0.25	Field isolate	Primary	in, io, dw, sp, aer[c]
Hitchner B1	Lentogenic	0.2	Field isolate	Primary	in, io, dw, sp, aer, bd
V4	Asymptomatic enteric	0.0	Field isolate	Primary	in, io, sp, aer, oral
Strain H[d]	Mesogenic	1.4	Attenuated by passage in eggs	Secondary	im, sc
Mukteswar[d]	Mesogenic	1.4	Attenuated by passage in eggs	Secondary	im, sc
Roakin[d]	Mesogenic	1.45	Field isolate	Secondary	im, ww

[a]ICPI, intracerebral pathogenicity index.
[b]aer = aerosol, bd = beak dipping, dw = drinking water, im = intramuscular, in = intranasal, oral = in food, sc = subcutaneous, sp = coarse spray, ww = wing web.
[c]These vaccines may give severe reactions when given by aerosol.
[d]Although still used in areas in which vND is endemic mesogenic vaccines fall within the OIE definition of viruses that cause vND.

Live Vaccines

Virus Strains. It is convenient to divide live NDV vaccines into two groups, lentogenic and mesogenic (Table 3.5). Note that the mesogenic vaccines fall within the current OIE definition of virus responsible for vND. They are used only in countries where vND is endemic and are suitable for secondary vaccination of birds because of their virulence. Even within the lentogenic group, however, is a considerable range in virulence, as demonstrated by Borland and Allan (47) who developed a stress index test to assess the potential effects of vaccines on susceptible chickens. The immune response increases as the pathogenicity of the live vaccine increases (206). Therefore, to obtain the desired level of protection without serious reaction, vaccination programs are needed that involve sequential use of progressively more virulent viruses or live virus followed by inactivated vaccine. Commonly used live vaccines and their pathogenicity indices for chickens are listed in Table 3.5.

Application of Live Vaccines. The objective of live vaccines is to establish an infection in the flock, preferably in each bird at the time of application. Individual bird treatments such as intranasal instillation, eyedrop, and beak-dipping are often used for lentogenic vaccines. Mesogenic vaccines usually require inoculation by wing-web stabbing or intramuscular injection.

The main appeal of live vaccines is that they may be administered by inexpensive mass application techniques. Probably the most common method of application used worldwide is via the drinking water. Generally, water is withheld from the birds for a number of hours and then vaccine is applied in fresh drinking water at concentrations carefully calculated to give each bird a sufficient dose. Addition of vaccine to header tanks has also been used successfully. Drinking water application must be carefully monitored as the virus may be inactivated by excessive ambient heat, impurities in the water, and even the type of pipes or vessels used to distribute the drinking water. To some extent, virus viability can be stabilized by the addition of dried skim milk powder to the drinking water (90).

Mass application of live vaccines by sprays and aerosols is also very popular due to the ease with which large numbers of birds can be vaccinated in a short time. It is important to achieve the correct size of particles by controlling the conditions under which the aerosol is generated (27, 170). Aerosol application usually is limited to secondary vaccination to avoid severe vaccine reactions. Coarse sprays of large particles do not penetrate deeply into the respiratory tracts of birds and give less reaction, so these may be more suitable for the mass application of vaccine to young birds. Coarse spraying of chicks at 1 day old may result in the establishment of infection in the flock with the vaccinal virus despite maternally derived immunity. It is believed, however, that in these circumstances, infections are established by the nasal or ocular route as a result of head rubbing on the backs of other birds and not necessarily directly by the spray (170). Aerosol and coarse-spray generators are available commercially (150); in the United States, a cabinet for coarse-spraying of day-old chicks is widely used (92).

A vaccine, based on the Australian V4 virus, has been developed specifically for use in village flocks in tropical countries. The recommended method of administration of the vaccine is in coated, pelleted feed. Initial laboratory and field trials suggest that this method is efficacious (69), but later studies have recorded problems probably related to the type of feed used as a vehicle (185, 234).

Advantages and Disadvantages of Live Virus Vaccination. Live vaccines usually are sold as freeze-dried allantoic fluid from infected embryonated eggs, are relatively inexpensive and easy to administer, and lend themselves to mass application. Local immunity is stimulated by infection with live viruses, and protection occurs very soon after application. Vaccine viruses may spread from birds that have been successfully vaccinated to those that have not.

Several disadvantages exist, the most important of which is that the vaccine may cause disease, depending upon environmental conditions and the presence of complicating infections. Therefore, it is important to use extremely mild virus for primary vaccination and, as a result, multiple applications of vaccine(s) usually are needed. Maternally derived immunity may prevent

successful primary vaccination with live virus. Although the ability of vaccinal virus to spread may be an advantage within the flock, spread to susceptible flocks, especially on multiage sites, can cause severe disease problems, particularly if dual infections with exacerbating organisms occur. Live vaccines may be killed easily by chemicals and heat and, if not carefully controlled during production, can contain contaminating viruses.

Inactivated Vaccines

Production Methods. Inactivated vaccines are usually produced from infective allantoic fluid treated with b-propiolactone or formalin to kill the virus and then mixed with a carrier adjuvant. Early inactivated vaccines used aluminum hydroxide adjuvants, but the development of oil-emulsion-based vaccines proved a major advancement. Different oil-emulsion vaccines vary in their formulation of emulsifiers, antigen, and water-to-oil ratios; most now use mineral oil (73).

Various seed viruses used in the production of the oil-emulsion vaccines include Ulster 2C, B1, La Sota, Roakin, and several virulent viruses. The selection criterion should be the amount of antigen produced when the virus is grown in embryonated eggs. Apathogenic viruses grow to the highest titers (101); therefore, it would seem an unnecessary risk to use a virus virulent for chickens.

One or more other antigens may be incorporated into the emulsion with NDV, and bivalent or polyvalent vaccines may include infectious bronchitis virus, infectious bursal disease virus, egg drop syndrome virus (Adenovirus 127), and reovirus (170).

Application of Inactivated Vaccines. Inactivated vaccines are administered by injection, either intramuscularly or subcutaneously.

Advantages and Disadvantages of Inactivated Vaccines. Inactivated vaccines are far easier to store than viable vaccines. They are expensive to produce and to apply because of the labor needed for their application. The labor expense can be partly offset by the use of polyvalent vaccines. Inactivated oil-emulsion vaccines are not as adversely affected by maternal immunity as live vaccines and can be used in day-old chicks (51). Some countries—e.g., the United States—have imposed a 42-day withdrawal period for oil-emulsion vaccines used in birds for human consumption. This restriction would limit the use of inactivated vaccines in some production sectors. Quality control of inactivated vaccines is often difficult, and mineral oils may cause serious problems to the vaccinator if accidentally injected (239). The major advantages of inactivated vaccine are the very low level of adverse reactions in vaccinated birds; the ability to use them in situations unsuited for live vaccines, especially if complicating pathogens are present; and the extremely high levels of protective antibodies of long duration that can be achieved.

Vaccination Programs

Vaccination programs and vaccines may be controlled by government policies. They should always be tailored to suit the prevailing disease situation and other factors, which include availability of vaccine, maternal immunity, use of other vaccines, presence of other organisms, size of the flock, expected life of the flock, available labor, climatic conditions, past vaccination history, and cost.

Timing of vaccination of broiler chickens can be especially difficult due to the presence of maternal antibodies. Because of their short life, broiler chickens are sometimes not vaccinated in countries where there is a low risk of ND.

Vaccination of laying hens always requires more than one dose of vaccine to maintain immunity throughout their lives (27). Actual programs depend on local conditions. In many countries, local customs or circumstances result in too little vaccination, overvaccination, or mistiming of vaccination, all of which may have serious consequences. The problems and pressures that may face the poultry farmer in tropical developing countries can frequently result in what has been described as "vaccine abuse" (119).

Interpretation of Vaccine Response

For NDV, the immune response is usually estimated by the HI titers obtained. Single vaccination with live lentogenic virus will produce a response in susceptible birds of about 2^4 to 2^6, but HI titers as high as 2^{11} or more may be obtained following a vaccination program involving oil-emulsion vaccine. The actual titers obtained and their relationship to the degree and duration of immunity for any given flock and program are difficult to predict. Allan *et al.* (27) presented predictions of the outcome of challenge of vaccinated young chickens with highly virulent NDV.

Vaccination of Other Poultry

Although vaccines developed primarily for chickens may be used effectively in other species, some differences in response may be apparent. For example, turkeys generally show a lower response, and as a result, they are often vaccinated first with La Sota followed by oil-emulsion vaccine (52). Some evidence exists, however, that La Sota may cause reaction in the respiratory tract (170), and that aerosol vaccination with lentogenic viruses causes pathologic lesions of the trachea (1). Considerable investigation is still being done into vaccination programs involving live and inactivated vaccines for use in turkeys (139, 153).

Guinea fowl and partridges have been successfully vaccinated with La Sota and/or oil-emulsion vaccines. Considerable investigation into the most suitable vaccines and regimens for pigeons has taken place due to the panzootic occurring in these birds during the 1980s (249).

Vaccination of ostriches and other ratites is not as well understood as other poultry, but doses and regimens have been suggested (10).

Future Developments

Molecular biology technology has enabled a much greater understanding of the pathogenicity (213) and antigenicity (214) of NDV and enabled cloning of the genes most closely involved (178). Groups working in this area have reported protective immunization with the HN gene expressed in recombinant fowl pox virus (48) and recombinant avian cells (64); or the F gene expressed in recombinant fowl pox virus (49, 242), vaccinia virus

(175), pigeon pox virus (159), and turkey herpesvirus (181, 220); or both HN and F expressed in fowl pox virus (143).

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Avian Metapneumovirus

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Definition and Synonyms

The clinical diseases that may result from avian metapneumovirus (aMPV) infections of turkeys or chickens have been termed turkey rhinotracheitis (TRT), swollen head syndrome (SHS), and avian rhinotracheitis (ART) based on clinical signs and lesions. However, these clinical signs and lesions are not specific for aMPV infections and can be confused with disease resulting from infections with other organisms such as *Bordetella avium, Ornithobacterium rhinotracheale* (ORT), infectious bronchitis virus (IBV), *Mycoplasma* species and other respiratory pathogens. Nevertheless, it is now universally accepted that the conditions referred to as TRT, SHS or ART can occur as a result of infection with aMPV. The more severe form of associated disease probably results from dual or secondary infection with other organisms, and for SHS, the characteristic "swollen head" appears as a result of infection with secondary adventitious bacteria, usually *Escherichia coli.*

Economic Significance

Avian metapneumovirus infections of poultry are associated with serious economic and animal welfare problems, particularly in commercial turkey flocks. Even in countries where vaccination against avian pneumovirus has become routine practice, the disease is still considered to be the most significant respiratory disease of turkeys, except for avian influenza (59). Since the initial outbreaks of aMPV infection in turkeys in the USA during 1997 losses in production have caused serious economic problems. It has been estimated that from 1997 to 2002 the losses to the Minnesota turkey industry were $15 million dollars per year (85). In commercial chickens the disease has less of an economic impact although in countries where infection is associated with SHS and losses in egg production aMPV can have a serious economic impact.

Public Health Significance

Although a similar metapneumovirus has been associated with upper respiratory disease in humans (hMPV) (102) there is no public health risks associated with aMPV infections in poultry.

History

Avian metapneumovirus was first described as a disease entity in turkeys in South Africa in the late 1970's (13). Later the dis-

ease was reported from Europe and the etiological agent was isolated at about the same time in the United Kingdom and France (73). A further two years passed before the agent responsible for the disease was characterised as a virus belonging to the family *Paramyxoviridae* and initially placed in the genus *Pneumovirus* (21). At about the same time that aMPV appeared in Europe and the Middle East a disease of chickens was observed which consisted of upper respiratory tract signs followed by a small number of flocks exhibiting swollen heads. This disease was known as "swollen head" syndrome (SHS) and was shown to be associated with a pneumovirus identical to what was then referred to as turkey rhinotracheitis (TRT) virus (1). During the early 1990s vaccines were developed for use in turkeys and chickens originating from the primary turkey isolates. However, by 1994 it was shown that two subtypes of aMPV existed, termed A and B (55) and it was subsequently shown that vaccines developed from a subtype A strain could confer protection against subtype B viruses (23). During the mid-1990s serological evidence of aMPV was reported from the Far East, often associated with SHS in chickens (23) and subtype A viruses were detected in chickens in Brazil in 1995 (7) and in turkeys in 2004 (34). In 1997 aMPV was described in turkeys in the United States for the first time (88) and was later shown to be antigenically distinct from subtypes A and B (86). All subsequent isolates from turkeys in North America have been shown to be antigenically similar and are referred to as subtype C aMPVs. Interestingly the disease has not been observed in chickens in North America and although it first appeared in Colorado, infections by aMPV now appears to be confined to turkeys in the states of Minnesota, Iowa, Wisconsin and the Dakotas based on ELISA serology (12).

Viruses similar to subtype C, but of a different genetic lineage, have also been reported to occur in Muscovy ducks in France associated with respiratory signs and egg production problems (98).

In a more recent report from France retrospective molecular analysis of viruses isolated from turkeys in the 1980s indicated the presence of a fourth subtype of aMPV, designated subtype D (9,99).

Subtype A viruses have also been detected in pheasants in the U.K. associated with respiratory disease problems (42,105).

Etiology

Classification

Avian metapneumoviruses are members of the subfamily *Pneumovirinae*, belonging to the family *Paramyxoviridae*. The subfamily consists of two genera; *Pneumovirus* consisting of mammalian respiratory syncytial viruses and mouse pneumovirus, and *Metapneumovirus* in which avian pneumoviruses are placed (84). Until recently it was thought that aMPV was the sole member of the *Metapneumovirus* genus but recent reports have indicated that similar viruses have been detected in humans in several countries associated with respiratory tract infections (64, 102). Avian metapneumoviruses have been further classified into four subtypes (A, B, C and D) based on nucleotide and deduced amino acid sequence data (33).

Morphology

Negative contrast electron microscopy of avian metapneumovirus reveals pleomorphic fringed particles, usually roughly spherical, of 80–200nm in diameter, although occasionally round particles with diameters of 500nm or more can be seen (Fig. 3.1). Fringed filamentous forms 80–100nm in diameter and up to 1000nm long may also be present (Fig 3.2), particularly in preparations from organ culture propagation. Collins and Gough (20) reported the surface projections to be 13–14nm in length and the helical nucleocapsid to be 14nm in diameter with an estimated pitch of 7nm per turn.

Chemical Composition

The virus genome is non-segmented and composed of single-stranded negative sense RNA of approximately 14 kilobases. In sucrose gradients the bouyant density of an isolate from turkeys was 1.21g/ml with an approximate molecular weight of 500 x 10^6. The same virus was shown to have about eight structural polypeptides of which two were glycosylated and three non-structural virus-specified proteins (20). These have been identified as nucleoprotein (N), phosphoprotein (P), matrix protein (M), fusion protein (F), second matrix protein (M2),small hydrophobic protein (SH), surface glycoprotein (G) and a large, viral RNA-dependent RNA polymerase (L), flanked by a leader and trailer at the 3′ and 5′ ends, respectively (58).

Virus Replication

Few detailed studies on aMPV have been published but virus mechanisms are thought to be similar to related negative strand viruses such as respiratory syncytial virus (RSV). Study of RSV has shown that virus replication and transcription both start at the 3′ leader sequence (37). Because of polymerase dissociation, genes are transcribed and expressed at reduced levels ongoing from the leader to trailer of the genome. The development of a reverse-genetic system for aMPV (76) has confirmed that as for RSV, the minimum replicative unit is the ribonuclear complex which comprises the nucleocapsid, phosphoprotein and M2 protein together with the viral polymerase. This study showed that the SH and attachment proteins can be deleted to leave viable viruses, at least in tissue culture. This suggests that the fusion protein can also play a role in virus attachment in addition to glycoprotein G.

Susceptibility to Chemical and Physical Agents

Early studies with one of the first European turkey isolates of aMPV reported that the virus was sensitive to lipid solvents, stable at pH 3.0 to 9.0 and inactivated at 56°C after 30 minutes (21). More recently, studies with a subtype C strain of aMPV isolated from turkeys in Minnesota, USA reported a similar resistance to pH 5 to 9 for one hour. In addition, the study reported that the isolate had lost viability by 12 weeks at 4°C, 4 weeks at 20°C, 2 days at 37°C and 6 hours at 50°C. Several disinfectants were effective in reducing the viability of the virus; including quaternary ammonia, ethanol, iodophor, a phenol derivative and sodium hypochlorite (bleach). Surprisingly, after 7 days of drying at room temperature the aMPV isolate remained viable (100). The survival of an aMPV subtype C isolate in built-up turkey litter at

different temperature conditions has also been studied. The results from the study indicated that virus could survive for up to 60 days at a temperature of –12°C and viral RNA was still detectable in the litter kept at 8°C after 90 days (104).

Strain Classification

Early studies using cross neutralization, ELISA techniques and polypeptide profiling suggested there were few strain differences between European isolates of aMPV (8, 39). However, more detailed studies using monoclonal antibodies (mAbs) produced to a variety of isolates demonstrated considerable antigenic differences between strains (22, 28). Further confirmation of subtype differences was obtained following nucleotide sequence analysis of the G (attachment) glycoprotein which showed that two subgroups designated A and B existed within the one serogroup (55). Subtype A and B viruses have been isolated from, and can infect, both chickens and turkeys (23).

More recent aMPV isolates from turkeys in Colorado and Minnesota, USA, were shown to have no significant serological relationship with subtype A and B strains from Europe using neutralization tests with mono-specific and monoclonal antibodies (30). Further detailed molecular analyses of the N, P, M, F and M2 protein genes of European viruses and isolates from the USA revealed that subtype C viruses had over 90% nucleotide sequence identity within the five genes compared to between 40 and 70% sequence identity with subtype A and B viruses (92). Phylogenetic analyses of three subtypes (A, B and C) showed that A and B viruses were more closely related to each other than either were to subtype C viruses (86). Retrospective analysis of two viruses isolated in France in 1985, subsequently referred to as subtype D, showed differences in the G gene sequence from subtypes A, B and C isolates (9). Phylogenetic comparison of the four subtypes has shown that A, B, and D viruses are more closely related to each other than to subtype C viruses (5, 9, 99).

Laboratory Host Systems

Initial problems in the laboratory diagnosis and determination of the etiology of aMPV were due primarily to a lack of suitable laboratory propagation system. The infectious nature of the disease could be demonstrated by typical clinical signs appearing in susceptible turkey poults placed in contact with infected birds or inoculated with filtered mucus from affected birds (2).

Inoculation of infective mucus into the yolk sac of turkey or chicken embryos resulted in embryo mortality after 4 or 5 passages, but virus was demonstrated to be at a very low titer (2). Similarly, inoculation of turkey or chicken tracheal organ cultures (TOCs) resulted in ciliostasis, but again, virus only replicated to low titers (38,69). Isolates adapted to embryos or TOCs, however, were capable of replication in cultures of primary chicken and turkey embryo cells, mammalian cells such as VERO, BS-C-1 and MA104 cells, with a characteristic cytopathic effect of syncytium formation and relatively high virus titers. A continuous quail tumour cell line (QT-35) has also been used to propagate the virus (43).

Subtypes A and B can be isolated in TOC but subtype C viruses have only been isolated in cell cultures or fertile eggs.

Pathogenicity

Despite the high morbidity and occasionally high mortality associated with aMPV in the field, the pathogenicity of aMPV isolates has been difficult to assess in the laboratory. Experimentally, infected birds often show recognizable signs of rhinotracheitis, but these are milder than those seen in the field (109). Chickens show, at most, only mild respiratory disease in laboratory infections and nasal mucus may only be discernible after light squeezing behind the nostrils. Transmission studies in two-week-old broiler chicks with a turkey isolate of aMPV from Minnesota produced clinical signs of coughing and sneezing for up to 8 days post inoculation. Tissue and intestinal samples from the broilers were positive by PCR for up to 9 days post inoculation (89). An isolate of aMPV from chickens with SHS was able to produce rhinotracheitis in infected turkey poults (83). Presumably, the difference in pathogenicity between laboratory and field infections is related to the conditions under which the birds are kept and the presence or absence of exacerbative organisms. In laboratory studies concurrent infection of turkey poults with aMPV and respiratory bacteria, such as *E coli*, *Bordetella avium* and *Ornithobacterium rhinotracheale* (27,49,68), *Mycoplasma gallisepticum* (74) and lentogenic Newcastle disease (101), significantly exacerbate and prolong clinical signs and morbidity. In experimentally infected turkey poults pre-infection with *Chlamydophila psittaci* was also shown to significantly increase the severity of aMPV infection (103).

Pathobiology and Epidemiology

Incidence and Distribution

Apart from Australasia all major poultry rearing regions of the world have reported the presence of aMPV. Evidence for the presence of the virus in commercial poultry has frequently been based on serological evidence alone (23,33). Due to difficulties in identifying or detecting the virus the number of countries reporting the isolation and characterisation of the virus are relatively few. Although aMPV has been reported in turkeys in the United States since 1997 there have been no reports on the isolation or detection of the virus in commercial chickens, even though there is evidence of the disease in neighbouring countries (50).

Natural and Experimental Hosts

Turkeys and chickens, apparently of any age, are known natural hosts of aMPV.

Additionally, Picault *et al.* (82) found aMPV antibodies in flocks of guinea fowl (*Numida meleagris*) and were able to produce a rhinotracheitis-like disease in this species with virus isolated from aMPV affected turkeys. Swollen head syndrome (SHS) has also been reported in guinea fowl (61) and pheasants (78) associated with aMPV. In the United Kingdom aMPV has been detected in pheasants with respiratory signs and serological studies suggest that the virus is widespread in game birds (42,105). Avian metapneumovirus has been detected by RT-PCR and occasionally virus isolation, in nasal turbinates of sparrows, ducks, geese, swallows, gulls and starlings sampled in the north

central region of the United States and shown to be closely related to subtype C viruses isolated from commercial turkeys (11,12,90,91). Subtype C-like viruses have also been reported to occur in commercial Muscovy ducks associated with respiratory signs and egg production problems (98).

In experimental infections with a subtype A aMPV Gough *et al*. (40) demonstrated susceptibility with clinical signs in turkeys, chickens and pheasants and an immune response to the virus in guinea fowl, although pigeons, geese and ducks appeared to be refractory to the virus. Antibodies to aMPV have been reported in farmed ostrich in Zimbabwe (15) and also in seagulls in the Baltic (47). In transmission studies in mice, rats and waterfowl using a turkey aMPV isolate from Minnesota, virus was detected for up to 14 days in mice and 6 days in rats. No clinical signs were observed in the waterfowl, but viral RNA was detected by PCR for up to 21 days post infection (72).

Transmission

The infectious nature of aMPV was established by contact transmission from affected to susceptible turkey poults, or by inoculation with filtered or unfiltered mucus, nasal washings or other materials from the respiratory tract of affected birds (2,69). Cook *et al.* (27) demonstrated that the virus was transmissible from infected to susceptible turkey poults placed in direct contact for a 9-day period after infection. These authors stressed the apparent importance of direct contact as, in their experiments, virus failed to spread to susceptible birds housed in the same room but in a different pen. Similar results have been reported by Alkhalaf *et al.* (3)using a subtype C aMPV. There is no published evidence that aMPV can be vertically transmitted even though high levels of virus can be detected in the reproductive tract of laying birds (53,57).

In most countries where aMPV has appeared as a new disease, it has spread rapidly. For example, in the United Kingdom, the disease was reported from most of the turkey-producing areas of England and Wales within 9 weeks of the first outbreak of the disease (1,50). The methods by which such spread takes place are unclear and, even on a single site, spread is unpredictable. Contaminated water, movement of affected or recovered poults, movement of personnel and equipment, feed trucks, etc. have all been implicated in outbreaks, while airborne spread or vertical transmission also have been put forward as possibilities. At present, only contact spread has been confirmed. The fact that North America remained free of aMPV for so many years when the disease was endemic in South and Central America, Europe and other parts of the world suggests that direct contact is important for transmission and spread of avian pneumoviruses. This view is supported by the situation in the US, in which the disease is widespread in Minnesota but has not spread significantly to other turkey producing areas or into commercial chickens. It seems highly likely that the density of the poultry population, particularly turkeys, will have a significant influence on the degree of spread of the virus (50). Migratory birds, particularly wildfowl, have been suggested as transmitters of aMPVs, although evidence of infection in wild species does not necessarily confirm this. Minnesota, the epicentre of subtype C aMPV infection in the USA lies under major wildfowl flyways which reach from Canada down to Central and South America. However, there has been no apparent spread of subtypes A and B viruses from South and Central America to the USA and no evidence of subtype C viruses spreading south.

Clinical Signs, Morbidity, and Mortality

The disease in turkeys has been described in detail elsewhere (23,60,73,93). Much of the reported variation in the clinical signs observed is attributed to management factors; such as overstocking, poor ventilation and damp conditions; or the presence of exacerbating adventitious agents that frequently occur with aMPV infections. Typically, clinical signs in young poults include snicking, rales, sneezing, nasal discharge, foamy conjunctivitis, swollen infraorbital sinus and submandibular edema. Coughing and head shaking are frequently observed particularly in older poults. In laying birds there may be a drop in egg production of up to 70% with an increased incidence of poor shell quality and peritonitis (53). Coughing associated with lower respiratory tract involvement may lead to prolapses of the uterus in breeding turkeys. When disease is seen, morbidity in birds of all ages is usually described as up to 100%. Flock mortality ranges from as low as 0.4% to as high as 50%, particularly in fully susceptible young poults. In uncomplicated infections recovery usually occurs between 10 and 14 days.

Avian metapneumovirus infection in chickens is less clearly defined and may not always be associated with clinical signs (23,51). The virus has been associated with swollen head syndrome (SHS) in chickens, which is characterized by the following clinical signs: swelling of the periorbital and infraorbital sinuses, torticollis, cerebral disorientation and opisthotonous, as a result of secondary *E. coli* infection. Usually less than 4% of the flock are affected although widespread respiratory signs are usually present. Mortality rarely exceeds 2% and in broiler breeders egg production is frequently affected. In commercial layers aMPV infection may also affect the quality of eggs (23,50). In laboratory studies the intravenous route of infection had a significant effect on the severity of clinical signs and egg production compared to the oculonasal route, in which egg production remained normal(31,48). There is evidence that infectious bronchitis virus together with *E. coli* may also be associated with SHS (35,71).

Pathology

Gross Lesions

Experimental infection of susceptible 5-week-old turkeys with a European isolate of aMPV resulted in complete deciliation of the trachea by 96 hours after infection (52). Following infection of laying turkeys a watery to mucoid exudate was found in the turbinates one to 9 days post infection, with excess mucus in the trachea (53). Various reproductive tract abnormalities were also reported by the same authors, including egg peritonitis, folded shell membranes in the oviduct, misshapen eggs, ovary and oviduct regression and inspissated albumin and solid yolk. Hens in lay may also present with prolapsed oviducts due to violent coughing. During natural field outbreaks, exacerbated by secondary pathogens, a variety of gross lesions have been described including airsacculitis, pericarditis, pneumonia and perihepatitis (23,41,51,87,93)

In aMPV infected chickens the only significant lesions noted

are those associated with SHS in broilers or broiler breeders. The major gross lesions include extensive yellow gelatinous to purulent edema in the subcutaneous tissues of the head, neck and wattles. Varying degrees of swelling of the infraorbital sinuses may also be seen (45,51,62,94).

Similar gross lesions have been reported to occur in commercially raised pheasant poults in Japan (78)

Microscopic

Detailed histological studies have been performed in experimentally inoculated turkey poults (66,73). At one to two days, post mortem examination of the turbinates revealed increased glandular activity, focal loss of cilia, hyperemia and mild mononuclear infiltration of the submucosa. Between 3 and 5 days damage to the epithelial layer and a copious mononuclear inflammatory infiltration in the submucosa was observed. Some transient lesions may also be seen in the trachea.

Experimental infection of chickens with turkey and chicken isolates have produced similar histological findings and these have been presented in detail elsewhere (16,66,67). In summary, the results provide clear evidence that aMPV can cause damage to the upper respiratory tract of chickens, although the damage is localized and transient.

Immunity

Active Immunity

Cell-Mediated Immunity. The results of laboratory studies suggest that the cell-mediated immune response provides the main resistance to infection of the respiratory tract with aMPV (56). Jones *et al.,* (54) showed that aMPV vaccinated, chemically bursectomised poults that were unable to seroconvert were still resistant to challenge with a virulent strain of aMPV.

Humoral Immunity. Many authors have described the humoral immune response of turkeys to aMPV infection, and these have been reviewed (73). Antibodies can be detected by ELISA, virus neutralization (VN) and indirect immunofluorescence. However, none of these tests are immunoglobulin specific. Following infection of turkeys aMPV antibodies were detected as early as 7 days post infection by ELISA and VN tests and were maintained for up to 89 days, when the trial was terminated (53).

Passive Immunity

Hens with aMPV antibodies will pass these to their progeny via the egg yolk. Titers will be directly related to the levels of circulating antibody in the parent bird. There is evidence that the presence of high levels of maternally derived antibody in one-day-old turkey poults will not prevent clinical disease following challenge with aMPV (75).

Diagnosis

Isolation and Identification of Avian Pneumovirus

Initially, virus isolation proved extremely difficult due to the fastidious nature of the virus, the frequency with which other secondary organisms could be isolated, and the timing of virus isolation attempts (41,50,60). Successful virus isolation was achieved in chicken or turkey embryos or chicken tracheal organ cultures and these have been used on a routine basis. More recently molecular techniques particularly RT-PCR tests have been developed to detect the virus.

Choice and Timing of Samples for Isolation

Although virus has been isolated from trachea, lung and viscera of affected turkey poults, by far the most fruitful source of virus has been ocular/nasal secretions or tissue scraped from the sinuses/turbinates of affected birds. It is extremely important to obtain samples as early as possible after infection as virus may only be present in the sinuses and turbinates for 6 to 7 days at the most (33,40,50). Isolation of virus is rarely successful from birds showing severe signs; presumably the extreme signs are a result of secondary, adventitious bacterial infections in birds predisposed by earlier virus infection. This probably accounts for the lack of success in isolating virus from chickens with SHS, as the characteristic signs appear to be due to secondary *Escherichia coli* infection. Furthermore, for reasons that are unclear, virus isolation from chickens appears to be more difficult than from turkeys.

Due to the labile nature of the virus it is essential that samples requiring despatch to a diagnostic laboratory for attempted virus isolation are sent immediately on ice (41,50). Where delays in despatch are unavoidable samples should be frozen at –50 to 70°C.

Virus Isolation

The various methods used for the primary isolation of aMPV have been published in detail elsewhere (23,33,41,43,51). What is apparent from the European and more recently, the American experience, is that a multiple approach to diagnosis should be used in order to maximise the chances of successfully isolating the virus.

Tracheal Organ Cultures. These are prepared from turkey or chicken embryos shortly before hatching or 1- to 2-day-old chicks originating from aMPV antibody-free flocks. The cultures can be maintained for several weeks and following inoculation with samples they are observed for ciliostasis, which may take several passages before a consistent effect is observed (33). Some of the early isolates of subtype A and B viruses were isolated using this method. However, tracheal organ cultures were found to be unsuitable for the isolation of subtype C viruses as the isolates did not cause ciliostasis (30).

Culture in Embryonating Eggs. Six- to 8-day-old embryonating turkey and chicken eggs from aMPV-negative flocks have been used to isolate the virus following inoculation by the yolk-sac route. Usually, serial passage is required before the agent causes consistent embryo mortality. The method is therefore both time consuming and expensive, and may not be successful. However, this technique was used to isolate the original subtype C Colorado aMPV strain in 1997 (79,88) and more recently in the

outbreaks in Minnesota, U.S.A. This method was also used to isolate the original aMPV strain in South Africa in 1980 (13).

Cell Cultures. This method is not generally successful for the primary isolation of aMPV strains. Occasionally, chicken embryo cells and VERO cells have been used successfully (43). Where positive results were obtained multiple blind passages were required before the virus produced a consistent cytopathic effect (CPE). QT-35 cells have also proved suitable for the primary isolation of APV strains (43). Once adapted to growth in embryonated eggs and tracheal organ cultures the virus can be cultivated to high titers in a range of avian and mammalian cells. The virus produces a characteristic CPE with the formation of syncytia within seven days.

Virus Identification

The isolated virus shows a paramyxovirus-like morphology when examined by negative contrast electron microscopy (EM). Particles are pleomorphic, spherical (80–600nm) or filamentous (up to 1000nm). The surface projections are 13 to 14nm in length and the helical nucleocapsid that can sometimes be seen emerging from disrupted particles is 14nm in diameter with an estimated pitch of 7nm per turn (20,33).

The physiochemical properties of the isolated virus can be investigated to aid identification and these have been described in detail elsewhere (41).

Strains can be distinguished using monoclonal antibodies but more recently molecular methods have been developed based on differences in the nucleotide sequences of the attachment protein (G) and other protein genes (33).

Direct Detection of Viral Antigens

A number of assays have been developed to detect and demonstrate the presence of aMPV antigen in both fixed and unfixed tissues and smears. The most widely used are immunoperoxidase (IP), immunofluorescence (IF) and immunogold staining and these have been described in detail (1,33,53). These techniques have generally been applied during laboratory studies on the replication and pathogenesis of aMPV in turkeys and chickens, and have limited value as diagnostic tools.

Molecular Identification

In recent years significant advances have been made in the diagnosis of aMPV based on the development of RT-PCR techniques. There is no doubt that molecular techniques are significantly more sensitive and rapid than conventional virus isolation methods (17,23,33,41). An important consideration when using PCR methods is whether to use subtype specific or generic-type PCRs, designed to detect several if not all aMPV subtypes. In Europe, where all four subtypes of aMPV have been reported, this is clearly an important consideration, whereas in the USA where only subtype C viruses have been reported, it is of less importance. RT-PCR methods using primers targeted to the F, M, and G genes have been developed but are limited in specificity and may not detect all subtypes (33,41). PCR methods using primers directed to the conserved region of the N gene have been described with the ability to detect representative aMPV isolates of subtypes A, B, C and D (9). Using this method positive products can be further analysed to determine the subtype using subtype specific PCRs or by sequencing and restriction fragment length analysis. A variety of RT-PCR techniques have been developed and evaluated and these have been extensively reviewed elsewhere (18,33,63,77)

Serology

Due to difficulties in isolating and identifying aMPV serological methods have been developed to confirm infection in commercial poultry and other avian species. The ELISA is the most commonly employed method but other techniques include virus neutralization and indirect immunofluorescence (10,41). The serological response in chickens following infection or vaccination is often weak compared to the response in turkeys.

In common with all serological tests both acute and convalescent sera should be submitted for analysis. The sera should be heat treated at 56ºC for 30 minutes and if delays in testing are unavoidable stored at -20ºC.

ELISA

A variety of commercial and in-house kits have been developed for detecting aMPV antibodies (23,41,73). The test can be used for screening large numbers of sera but differences in sensitivity and specificity have been reported (23,70,97). This is principally due to variations in the antigenicity and purity of the antigen used to coat the ELISA plates. It has been shown that vaccinal antibodies may not be detected if heterologous strains of aMPV are used to prepare the coating antigen for the ELISA plates (36). Studies using ELISA kits incorporating subtype A or B antigens were found to be relatively insensitive for detecting antibodies to the Colorado strain of aMPV (30). Some competitive ELISA kits incorporate a specific aMPV monoclonal antibody that facilitates the testing of sera from different avian species. However, the kits were found to be unsuitable for the detection of antibodies against aMPV isolates from the US (30). More recently ELISAs have been developed and evaluated which incorporate whole virus antigens prepared from Colorado and Minnesota isolates of aMPV (4,19,65). More sensitive and specific ELISAs using M and N protein expressed antigens in sandwich-capture ELISAs for detecting subtype C antibodies have also been described (44).

Virus Neutralization

Antibodies to aMPV can be detected by standard neutralization techniques using sensitive cell cultures or tracheal organ cultures. The test in chicken embryo cells has been detailed elsewhere (41). Cross reactivity occurs with subtype A and B viruses although good correlation with ELISA and indirect immunofluorescence has been reported (10,33). However, the VN test is time consuming, expensive and unsuitable for mass serological screening of poultry flocks.

Fluorescent Antibody Test

A number of indirect immunofluorescent antibody tests have been described (33,73). These tests are useful as a research tech-

nique but have limited application for testing large numbers of poultry sera for aMPV antibodies.

Differential Diagnosis

Strain Variability

The morphological appearance of aMPV gives no indication of the strain or subtype. Subtype A and B viruses were originally differentiated on the basis of nucleotide sequence analysis of the attachment (G) protein gene (55) and mAB analysis (22,28), although they belong to the same serotype. With the emergence of subtype C in the USA, and evidence of subtypes C and D in France, it seems likely that further subtypes of aMPV remain undetected. It is also probable that current RT-PCR methods may fail to detect the presence of "new" subtypes of aMPV. Clearly a multi-diagnostic approach is required in order to detect further subtypes of aMPV, including virus isolation, electron microscopy and the development of more sensitive PCR methods (33).

Other Viruses

Paramyxoviruses, particularly Newcastle disease and APMV-3, infectious bronchitis, and influenza viruses may cause respiratory disease and egg production problems in chickens and turkeys that closely resemble aMPV infection. Paramyxoviruses and some avian influenza virions are similar in morphology but can be easily distinguished from aMPV as they possess hemagglutinin and neuraminidase activity. Infectious bronchitis virus can be differentiated from aMPV by morphological and molecular characteristics.

Bacteria and Mycoplasmas

A wide range of bacteria and *Mycoplasma* species can cause disease signs very similar to aMPV infection (50,59). These organisms often act as secondary opportunistic pathogens following aMPV infection and may cause considerable diagnostic problems. Only by isolating or identifying aMPV in the affected birds can a clear distinction be made.

Intervention Strategies

Management Procedures

It is now accepted that management factors will significantly influence the severity of aMPV infection in commercial poultry, particularly turkeys. Poor management practices such as inadequate ventilation and temperature control, high stocking densities, poor litter quality and general hygiene, multi-age stock and the presence of secondary pathogens can all exacerbate aMPV infection (41,51,59,93). Debeaking or vaccinating flocks at a critical time can also influence the severity of clinical signs and eventual mortality due to aMPV infection (6). As a general principle, good biosecurity is essential in preventing the introduction and spread of aMPV onto poultry farms as evidence suggests that wild birds may also act as carriers of the virus. Disinfection of delivery and catching crews, equipment and feed trucks should be routine practice.

Some success in reducing the severity of the disease by controlling secondary adventitious bacteria with antibiotics has been reported (46).

Vaccination Strategies

Both live attenuated and inactivated aMPV vaccines are available commercially for use in turkeys and chickens. Early work on the attenuation of the virus was difficult due to poor attenuation of the virus and problems in reproducing a suitable challenge model in the laboratory (96). However, there are now a number of reports on the successful attenuation of aMPV strains in a variety of cell cultures and their effective use as vaccines (14,24,25, 26,80,106). The live attenuated vaccines have been shown to stimulate both systemic immunity and local immunity in the respiratory tract (56). In turkeys and particularly chickens the humoral antibody response is poor following primary live vaccination but birds may still be protected to challenge via cell mediated immunity in the respiratory tract (63). Similarly, high levels of maternally derived antibody will not prevent young turkey poults from developing clinical disease following exposure to virulent aMPV (75). Studies have shown that good cross protection occurs following vaccination with subtype A and B vaccines (29) and that vaccines produced from both subtype A and B strains of aMPV will confer protection against the Colorado subtype C strain of virus (23). To produce complete protection in adult birds oil-adjuvanted inactivated aMPV vaccines are administered to birds previously primed with live vaccines. A typical vaccination program for aMPV in turkeys would be application of a live subtype A or B strain, or combined at day-old using a coarse spray, repeated at 7 to 10 days and again at 4 to 6 weeks. The strategy is to produce cell mediated immunity in the respiratory tract. Breeding stock would additionally receive inactivated vaccine at 16 to 20 weeks. Vaccination reactions may occur due to the presence of secondary pathogens.

There is evidence that live infectious bronchitis vaccine can interfere with the replication of aMPV vaccines in chickens (32).

Future Developments

Recombinant vaccines that incorporate specific immunogens, such as the fusion (F) glycoprotein, in fowl pox virus (108) and a DNA plasmid (95) have also been evaluated. From the results obtained with the poxvirus recombinant vaccine it was concluded that experimental vaccination induced aMPV antibodies in experimental turkeys and produced some protection to challenge (108). Studies in turkeys on the immunity to an aMPV vaccine following in ovo vaccination have also been reported. The results indicated that this route of vaccination has several advantages compared to conventional methods of vaccination (107). In a recent report a cold adapted strain of aMPV was evaluated as a vaccine and was shown to produce protection to challenge for up to 14 weeks post vaccination (81).

Subunit and deletion mutant vaccines are also being developed and evaluated.

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Avian Paramyxoviruses 2–9

D. J. Alexander and D. A. Senne

Introduction

The prototype strains of the eight other serotypes of avian paramyxovirus (APMV-2 to APMV-9) and their usual hosts are listed in Table 3.1.

Definitions and Synonyms

Very few synonyms have been used for the other avian paramyxoviruses. The term "Yucaipa" viruses has been applied to APMV-2 viruses, as the first isolate was APMV-2/chicken/California/Yucaipa/56, the prototype of the serogroup. Isolates of the APMV-5 serotype occasionally have been referred to as "Kunitachi" viruses, again after the prototype virus.

Economic Significance

The economic significance of avian paramyxoviruses other than Newcastle disease virus that infect poultry is that of viruses causing significant respiratory disease and egg production problems in laying birds. In uncomplicated infections of turkeys with APMV-3, this level of disease, especially in laying birds, has been considered to have sufficient economic impact to render the birds unprofitable and to justify the use of expensive inactivated vaccine (21, 28). However, the greatest contribution to disease and consequent economic impact is the exacerbative effect avian paramyxoviruses may have on other concomitant bacterial or virus infections. Lang *et al.* (37) considered that, in these circumstances, the disease was sufficiently serious to warrant "prompt disposal of the infected birds."

Public Health Significance

Although the ability of Newcastle disease viruses to infect humans has been well documented (see previous), no reports have been made of other APMV serotypes infecting humans. However, the potential may exist, and virus of APMV-2 serotype was isolated from cynomolgus monkeys (45).

History

Avian Paramyxovirus Type 2 (APMV-2)

In 1956, Bankowski *et al.* (17) isolated a paramyxovirus (27) from a chicken suffering from laryngotracheitis in Yucaipa, California. It was serologically distinct from Newcastle disease virus (NDV) and caused only mild respiratory disease in chickens. Serologic surveys of poultry in the United States indicated that this virus was widespread, more frequently infecting turkeys than chickens (18, 23). Subsequent investigations suggested that viruses of the same serotype were common in poultry around the world (4).

Testing during quarantine of imported caged birds since the early 1970s has frequently resulted in the isolation of APMV-2 viruses, primarily from passerines but also from psittacines (4, 49). Surveillance of wild birds during the 1970s often resulted in the isolation of APMV-2 viruses, most frequently from passerine species (4).

Avian Paramyxovirus Type 3 (APMV-3)

Paramyxoviruses representing a third serotype were isolated from turkeys in Ontario in 1967 and Wisconsin in 1968 and later detected serologically in turkeys in other states of the United States (52). Serologically related viruses have now been reported from turkeys in several countries in Europe.

APMV-3 viruses are also frequently isolated from captive caged birds in most countries where quarantine is imposed, most often from psittacine species, although passerines are also susceptible (4). There is evidence that these viruses differ antigenically from the turkey APMV-3 viruses (14).

Avian Paramyxovirus Types 4–9 (APMV–4 to APMV–9)

The initial isolations of other AMPV viruses were all made in the 1970s, those identified as APMV-4, APMV-6 and APMV-8 were as a result of surveillance studies of feral ducks and geese, usu-

ally for influenza viruses. APMV-4 and APMV-6 have been isolated from domestic ducks, but not APMV-8. APMV-9 serotype had consisted of a single isolate from domestic ducks in New York in 1978, until the isolation of a further member from a feral pintail duck (*Anas acuta*) in Italy in 2004 (24), which suggests this serotype probably also has wild bird reservoirs and worldwide distribution.

Etiology

Classification of Avian Paramyxoviruses

Information on the classification into order, family, subfamily and genus is given in the Introduction to this chapter.

Tumova *et al.* (53) suggested grouping avian paramyxoviruses on the basis of their antigenic relatedness in hemagglutination inhibition (HI) tests. The prefixes PMV-1, PMV-2, etc., were adopted to signify serotype, and the nomenclature proposed for naming influenza isolates (55) was used for the avian paramyxovirus isolates. Subsequently, the International Committee on Taxonomy of Viruses adopted the abbreviations APMV-1, APMV-2 etc. (47), and these abbreviations are used in this chapter (Table 3.1).

There has been no attempt to make more specific definition of a serotype, and further viruses have been grouped based on their relationships in HI tests. When neuraminidase inhibition (34, 35, 43, 52), serum neutralization (52) or agar gel diffusion (1, 11, 34, 36) tests have been used, however, similar groups have resulted.

Despite the consistency of the serologic groupings, some cross relationships exist between viruses of the different serotypes (4). Usually, these have been very minor, although Lipkind *et al.* (38, 40) considered them sufficient to suggest a phylogenic relationship between APMV-1, -3, -4, -7, -8, and -9 and between APMV-2 and -6. However, the relationship between APMV-1 and APMV-3 viruses appears to be closer and more important than the others.

Smit and Rondhuis suggested that there were low serologic reactions between NDV and APMV-3/parakeet/Netherlands/75 (51), which were later confirmed (6). In addition, prior infection of chickens with some APMV-3 viruses conferred protection against challenge with a virulent NDV strain (9). More recently, a monoclonal antibody against the pigeon variant APMV-1 inhibited APMV-3 viruses isolated from exotic birds in HI tests and bound to cells infected with these APMV-3 viruses (12, 26). Because turkey APMV-3 isolates also show relationships with APMV-1 viruses, but none could be demonstrated to react with this monoclonal antibody, other epitopes may be shared by the two serotypes.

Genetic data for APMV-2 to APMV-9 is extremely limited. Chang *et al.* (25) sequenced the entire genome of a APMV-6 isolate. Although the genome appeared to code for a SH protein, which was absent in APMV-1 viruses but present in members of the *Rubulavirus* genus, phylogenetic analyses placed the APMV-6 virus closer to APMV-1 than viruses from other *Paramyxovirinae* genera. Data was available for the hemagglutinin-neuramindase (HN) gene of APMV-2 and APMV-4 viruses and the fusion (F) gene of APMV-2, phylogenetic analyses of these genes showed APMV-1, -2, -4 and -6 to form a cluster within the *Paramyxovirinae* subfamily, i.e. the *Avulavirus* genus.

Morphology

Negative contrast electron microscopy of members of the *Avulavirus* genus reveals very pleomorphic virus particles. Generally, they are rounded and 100–500 nm in diameter, although filamentous forms of about 100 nm across and of variable length are often seen. The surface of the virus particle is covered with projections about 8 nm in length. In most electron micrographs of avian paramyxoviruses, the "herring bone" nucleocapsid, about 18 nm across, may be seen either free or emerging from disrupted virus particles.

Chemical Composition

The number and molecular weights of the structural polypeptides for the other avian paramyxoviruses are similar to those reported for Newcastle disease virus, although minor variations in molecular weights have meant that PAGE profiles could be used to show similarities between isolates that coincide with the serogroups (7). Variation has also been reported within serogroups, especially for APMV-7 viruses (13).

Virus Replication

This is assumed to be similar to Newcastle disease virus (see previous discussion).

Susceptibility to Chemical and Physical Agents

This is assumed to be similar to Newcastle disease virus (see previous discussion).

Strain Classification

For most of the APMV serogroups, too few isolates have existed to examine strain relationships and classification. Some work has been done in this area for three of the serotypes infecting poultry.

Avian Paramyxovirus Type 2

Considerable antigenic and structural diversity has been recorded among APMV-2 viruses (2, 7), but these have not been related to any epizootiologic or biologic properties. Ozdemir *et al.* (46) prepared three MABs with hemagglutination inhibition activity against the APMV-2 type species and were able to place the 53 isolates tested with them into four groups.

Avian Paramyxovirus Type 3

APMV-3 virus isolates also show considerable diversity. There appears to be antigenic differentiation between those isolated from exotic birds and those from turkeys. This has been confirmed by MABs to APMV-3/turkey/England/MPH/81. With the use of these, Anderson *et al.* (14) showed that although some antibodies reacted with isolates from either source, others bound specifically to turkey viruses. Turkey isolates from the United States and Germany were distinguishable from turkey

isolates from Great Britain and France and possibly more closely related to exotic bird isolates. This division of APMV-3 isolates into two groups was supported by studies with a MAB to an APMV-1 pigeon variant isolate that was also able to react with APMV-3 isolates from exotic birds but not with those from turkeys (26).

Avian Paramyxovirus Type 7

APMV-7 virus isolates have shown marked antigenic variation, and there has been some debate as to whether or not they should all be placed in the same group. Alexander *et al.* (13) examined the antigenic relatedness of six APMV-7 viruses isolated from pigeons and doves and recorded considerable variation placing the viruses in three groups. Two of the groups, consisting of 3 and 2 viruses, appeared to have little relatedness, but viruses in both showed close relationships with the sixth virus examined.

Laboratory Host Systems

As with Newcastle disease virus, embryonated chicken eggs are considered the best medium for the isolation and propagation of the other avian paramyxoviruses. For some serotypes, yolk sac or amniotic inoculation may be the route of choice for isolation or replication (31, 44).

Pathobiology and Epidemiology

Distribution

Avian Paramyxovirus Type 2

APMV-2 viruses are found in feral birds, chiefly passerines, in European, Asian, African, and American countries (3, 30), probably accounting for their common isolation from imported caged birds (3). Isolations from domestic poultry have been rare, although problems associated with such viruses have been recorded in the United States, Canada, the former Soviet Union, Japan, Italy, Israel, India, and France in chickens or turkeys (2, 3).

Avian Paramyxovirus Type 3

APMV-3 viruses have also been isolated from imported exotic birds and other birds held in captivity, but, unlike APMV-2 viruses, there have been no reports of APMV-3 viruses from feral birds (3). APMV-3 virus infections of domestic poultry have been restricted to turkeys in Canada and the United States (52), Great Britain (41), France (15), and Germany (57). There have been no reports of naturally occurring infections of chickens with APMV-3 viruses, although they are fully susceptible (8).

Natural and Experimental Hosts

The general groups of birds reported to be infected with the different serotypes are shown in Table 3.1 and in more detailed reviews (2, 4). Isolations of avian paramyxoviruses from different species have been rarely associated with specific disease episodes. APMV-3 viruses have been related to disease in certain psittacine species such as encephalitis with high mortality in parakeets of the *Neophema* and *Psephotus* genera (51), steatorrhea and pancreatic lesions in *Neophema* parakeets (54), and high mortality in lovebirds, *Agapornis roseicollis* (33). APMV-5 viruses appear to have a very limited host range being isolated only from budgerigars, *Melopsittacus undulatus,* in which infection resulted in high mortality (32, 43). Viruses antigenically related to APMV-7 viruses have been associated with deaths in snakes following respiratory and nervous disease signs (20).

Spread

Little information is available on the spread of other avian paramyxoviruses. For APMV-2 and APMV-3 serotypes, infection of poultry leads to shedding from the respiratory and intestinal tracts, so it is assumed that the methods of spread of NDV would also apply to these. APMV-2 viruses have been shown to infect feral passerines that may invade poultry houses, but, in the absence of any wild bird host for APMV-3 viruses, it seems most likely that this subtype has been introduced into different countries by the importation of infected poultry or by humans.

The prevalence of APMV-7 viruses in pigeons and doves, which frequently invade poultry houses or mingle with poultry kept outdoors, suggests that these may have been the source of the two reported outbreaks of APMV-7 in turkeys (48) and ostriches (56).

Incubation Period

Little work has been done on assessing the incubation period for the avian paramyxoviruses infecting poultry. Bankowski *et al.* (17) reported rales in chickens 4–6 days after they were inoculated intratracheally with APMV-2 virus. Tumova *et al.* (52) reported mild respiratory signs from day 2 after infection of adult turkeys with APMV-3 virus. Saif *et al.* (48) reported mild respiratory signs from day 2 in turkeys infected with the APMV-7 turkey isolate.

Clinical Signs

Avian Paramyxovirus Type 2

APMV-2 viruses have been associated with mild respiratory or inapparent diseases in chickens and turkeys (18, 23, 29). Unlike NDV, APMV-2 infections have been reported to be more severe in turkeys than chickens, and Lang *et al.* (37) reported severe respiratory disease, sinusitis, elevated mortality, and low egg production in turkey flocks infected with APMV-2 complicated by the presence of other organisms. APMV-2 viruses have been reported to be widespread in turkeys in Israel and associated with severe respiratory disease in complicated infections (39). In experiments conducted under field conditions, Bankowski *et al.* (19) demonstrated that APMV-2 infections of laying turkeys resulted in egg production losses with reduced hatchability and poult yield, but fertility was unaffected.

Avian Paramyxovirus Type 3

APMV-3 virus infections of domestic poultry appear to have been restricted to turkeys. Clinical signs are usually egg production problems, although these have been occasionally preceded by mild respiratory disease (10, 15, 16, 41, 52). Egg production usually declined rapidly with a large number of white-shelled eggs, although hatchability and fertility were rarely affected.

Avian Paramyxovirus Type 6

APMV-6 isolates have also been obtained from turkeys showing mild respiratory disease and egg production problems. Viruses of this serotype have been isolated frequently from domestic ducks in which the virus appears to be apathogenic (42, 50).

Avian Paramyxovirus Type 7

Saif *et al.* (48) reported the isolation of an APMV-7 virus from a series of outbreaks in turkeys. The clinical signs were primarily respiratory with elevated mortality (0.9%/wk) over a 4-week period. In laying hens, egg production was not affected significantly, but there was an increase in white-shelled eggs.

Diagnosis

Isolation and Identification of Causative Agent

The samples taken and methods involved for the isolation of other avian paramyxoviruses are identical to those for NDV. Inoculation of 6- to 7-day-old embryonated eggs via the yolk sac should also be considered, because greater success has been reported for this route with some viruses. APMV-5 viruses do not grow after inoculation into the allantoic cavity and require amniotic inoculation of embryonating eggs or propagation in primary cultures of chick embryo cells (43).

Serology

The same serologic tests used for NDV (APMV-1) can be used for the other avian paramyxoviruses. APMV-5 viruses have been reported as not agglutinating RBCs (43). Gough *et al.* (32) reported, however, the isolation of virus clearly related to APMV-5 viruses that agglutinated guinea pig RBCs well and chick RBCs to a lesser extent. Hemagglutination inhibition antibodies to APMV-3 viruses may be detected in turkeys and chickens showing high vaccine-induced titers to NDV, and ND-vaccinated birds infected with APMV-3 viruses show a rise in HI titer to both viruses (10, 22).

Differential Diagnosis

Viral HA activity may be due to any of the nine avian paramyxovirus serotypes or any of the 16 influenza type A hemagglutinin subtypes that are known to infect birds. Demonstration that the virus is of a specific serotype usually can be carried out by a simple HI test with specific polyclonal antisera.

Newcastle disease virus (APMV-1) shows some cross-reaction in HI tests with several of the other avian paramyxovirus serotypes, especially APMV-3 psittacine isolates, using polyclonal antisera (5). Although the potential for misdiagnosis largely can be eliminated by the use of control sera and antigens in conventional tests, the use of MABs in routine diagnosis can give an unequivocal result.

Intervention Strategies

Few, if any, countries have national control policies for the other avian paramyxoviruses, although, in some, vaccination is permitted for APMV-3 viruses. Despite the frequent isolation of APMV-2 and APMV-3 viruses from passerine and psittacine birds in quarantine (4), little usually is done to restrict the introduction of such birds.

At the farm level, bird proofing of poultry houses should greatly reduce the possibility of introducing paramyxoviruses such as APMV-2 by feral birds. Other preventive measures taken for NDV will apply equally to the other APMV types. Lang *et al.* (37) suggested depopulation for turkey flocks infected with APMV-2 virus if complicated by other organisms.

Management Procedures

Management procedures at the farm level pertaining to Newcastle disease, especially those relating to biosecurity, would also be effective at controlling infections by other avian paramyxoviruses.

Vaccination

The other avian paramyxoviruses do not cause overt disease with high mortality and, thus, their economic impact is considerably less than NDV. Nevertheless, the egg production problems that have been associated with infections of turkeys by APMV-3 viruses can be sufficiently severe to warrant vaccination; for several years, oil-emulsion vaccines to this serotype have been available in Europe and the United States (21, 28). They appear to be effective at preventing the serious egg production losses associated with APMV-3 infections in laying turkeys.

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Chapter 4

Infectious Bronchitis

David Cavanagh and Jack Gelb Jr.

Introduction

Infectious bronchitis (IB), also called avian infectious bronchitis, is a common, highly contagious, acute, and economically important viral disease of chickens caused by coronavirus infectious bronchitis virus (IBV). The virus is acquired following inhalation or direct contact with contaminated poultry, litter, equipment, or other fomites. Vertical transmission of the virus within the embryo has never been reported, but virus may be present on the shell surface of hatching eggs via shedding from the oviduct or alimentary tract. The disease occurs in all poultry-producing countries. The highly transmissible nature of IB and the occurrence and emergence of multiple serotypes of the virus have complicated control by vaccination. Adult poultry (e.g., layers) are the source of new, previously unrecognized serotypes, also referred to as variants. Infectious bronchitis has no known human health significance.

In young growing chickens, IBV infection causes respiratory disease. Reduced weight gain and feed efficiency are observed in meat-type broiler chickens. Infection also predisposes broilers to bacterial airsacculitis, pericarditis, and perihepatitis. Mortality, up to 30%, often peaks at five to six weeks of age during the last two weeks of the broiler's life. Immunosuppression will further exacerbate disease and increase mortality. Mortality is much lower (<1%), and recovery is faster in outbreaks due to mild strains of IBV or those in which bacterial involvement is limited.

Some strains of IBV are nephropathogenic and cause renal (kidney)-induced mortality of up to approximately 25% in susceptible flocks.

Egg production and quality declines are noted in layer and breeder chickens. The virus may replicate in the oviduct and cause permanent damage in immature females or pullets resulting in limited egg production later in life. Infection of hens during lay may reduce egg production up to 10% or more depending on the immune status of the flock. Egg shells are commonly malformed and more susceptible to breakage, due to thinning of the shell. Eggs from breeds with pigmented shells may be paler than normal. Albumen from affected eggs is of a watery viscosity. Egg production often recovers following field infections of vaccinated layers but may be permanently depressed in highly susceptible flocks.

Coronaviruses with protein sequences very similar to those of IBV have been isolated from pheasants (*Phasianus colchicus*), turkeys, guinea fowl (*Numida meleagridis*), partridge (Alectoris sp.), peafowl (*Pavo cristato*), and the non-gallinaceous teal (*Anas* sp.). As discussed later, evidence is accumulating that IBV has a wider host range than was previously believed (20). The turkey coronaviruses are described at length in Chapter 12.

History

Infectious bronchitis was first observed in the United States in North Dakota in 1930. A report by Schalk and Hawn in 1931 (51) of the clinical signs and preliminary laboratory studies of those cases is recognized as the first report of IB. Initially, IB was recognized as primarily a disease of young chicks. However, it was later observed to be common in semi-mature and laying flocks. Other manifestations of IB include egg-production declines in laying flocks, noted following the typical respiratory disease in the 1940s, and kidney lesions observed in the 1960s. The prevalence and economic importance of the disease resulted in efforts to prevent IB in laying flocks by controlling the exposure of chickens to IBV during the growing stage prior to the onset of egg production. This effort by Van Roekel in 1941 (59), which had some success, was the initial step toward the development of the vaccination programs used today.

Other early milestones include the establishment of the virus etiology by Beach and Schalm in 1936, the first cultivation of the virus in embryonating chicken eggs by Beaudette and Hudson in 1937, the development of the "H" vaccines—e.g., the ubiquitous H120 about 1960 (10), and the report in 1956 by Jungherr and colleagues that the Connecticut isolate of 1951 and the Massachusetts isolate of 1941 produced similar diseases but did not cross-protect or cross-neutralize (59). The Jungherr's report was the first demonstration that the etiology of IB included more than one serotype. More about the early history of IB research can be found in the review by Fabricant (59).

Incidence and Distribution

Infectious bronchitis is distributed worldwide. In the United States, several serotypes in addition to the originally identified Massachusetts (Mass) type of IBV have been identified, beginning in the 1950s (59, 93, 132). Mass-type strains have been isolated in Europe and Asia since the 1940s and up to the present, although other serotypes found in North America have not generally occurred on other continents. Dozens of other serotypes have been isolated in Africa, Asia, India, Australia, Europe, and South America (18–20, 31). Outbreaks of IB frequently have occurred, even in vaccinated flocks. The virus strains isolated from

those outbreaks are often, but not always, (137) found to be a serotype distinct from the vaccine type. Several serotypes can co-circulate in a given region.

Etiology

Classification

Infectious bronchitis virus is a member of the *Coronaviridae*, which includes two genera, *Coronavirus* and *Torovirus*. The *Coronaviridae* family is, together with the *Ateriviridae* and *Roniviridae*, within the order *Nidovirales* (58). IBV is in Group 3 of the Coronavirus genus, together with coronaviruses from other avian species (58, reviewed in 19). Groups 1 and 2 comprise mammalian coronaviruses that differ extensively from IBV with respect to genome organization and gene sequences. A torovirus has been implicated in enteric disease of turkeys (see Chapter 13).

Morphology

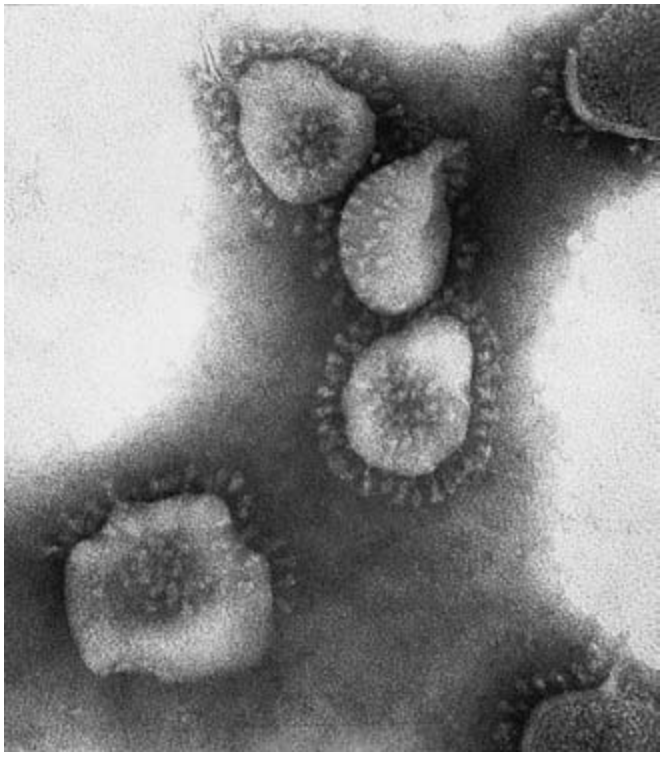
Infectious bronchitis virus is round to pleomorphic in shape. It possesses an envelope that is approximately 120 nm in diameter with club-shaped surface projections (spikes) about 20 nm in length (Fig. 4.1). The spikes are not packed as closely as the rod-shaped spikes of paramyxoviruses. Ribonucleoprotein (RNP or core) structures released from spontaneously disrupted particles could be visualized by shadowing but not by negative-staining. For the most part, the RNP was observed as strands of only 1–2 nm in diameter, but coiled structures of 10–15 nm diameter occasionally were observed. The appearances of toroviruses and coronaviruses in the electron microscope can be very similar (56).

Infectious bronchitis virus strains differ in their density in sucrose gradients; particles with a full complement of spikes have a density of 1.18 g/mL, and lesser-spiked particles may be as low as 1.15 g/mL. Centrifugation forces of greater than 100,000 g should be avoided, as loss of spikes, or at least the S1 subunit that is believed to form most of the globular head of the spike protein, can occur. Incubation at 37°C sometimes results in the loss of the S1 subunit (159). The S2 glycopolypeptide, which anchors the S protein in the membrane, is not linked to S1 by disulphide bonds.

Chemical Composition

Infectious bronchitis virus virions contain three major structural proteins (i.e., that are components of virus particles [virions]): the spike (S) and membrane (M) glycoproteins and the internal nucleoprotein (N) (58, 112). In addition, there are small amounts of a fourth protein (small membrane protein, E), which is essential for virus particle formation. The S protein comprises two or three copies of each of two glycopolypeptides, S1 and S2 (approximately 520 and 625 amino acids, respectively). Hemagglutination-inhibiting (HI) and most of the virus-neutralizing (VN) antibodies are induced by S1 (22, 86, 106), as is protective immunity (94)

Only about 10% of the M protein is exposed at the outer virus surface. The N protein surrounds the single piece of single-stranded, positive sense, RNA genome (to form the RNP) that comprises approximately 27,600 nucleotides, the whole of which has been cloned and sequenced for several strains. "Infectious clone" systems have been developed for IBV, enabling the manipulation of any part of the genome (16, 17, 167).



4.1. Virion of avian IBV illustrating club-shaped projections. Preparation negatively stained with phosphotungstic acid. ×3300,000. (Berry and Almeida)

Virus Replication

It is the S glycoprotein that is responsible for attachment of IBV to host cells. Indeed, experiments with recombinant IBV in cultured cells showed that the S protein is a determinant of host cell range (16). Whether S determines any tropisms within chickens and/or host range with regard to infection of other avian species remains to be demonstrated. The virus replicates in the cytoplasm, five subgenomic messenger RNAs being produced by a discontinuous transcription mechanism. Three of these mRNAs, 2, 4 and 6, are responsible for production of the S, M and N virion proteins, respectively, while the other two mRNAs, 3 and 5, encode three and two proteins, respectively. One of the gene 3-encoded proteins is the E protein; the other proteins encoded by genes 3 and 5 are nonstructural proteins that are not incorporated in virus particles. Genetic manipulation has shown that these four nonstructural proteins are not essential for replication but are probably helpful to the virus during replication *in vivo* (17, 78). New virus starts to appear 3–4 hours after infection, with maximum output per cell being reached within 12 hours at 37°C. Virions are assembled at internal membranes (e.g., Golgi membranes, not at the cell surface).

Resistance to Chemical and Physical Agents

Thermostability

Most strains of IBV are inactivated after 15 minutes at 56°C and after 90 minutes at 45°C. Long-term storage of IBV at –20°C should be avoided, but infectious allantoic fluid has remained viable after storage at –30°C for many years (reviewed in 21). Infected tissues stored in 50% glycerol are well preserved, and tissues in this medium can be shipped to a laboratory for diagnosis without refrigeration. Outdoors, survival up to 12 days in spring and 56 days in winter has been reported.

Lyophilization

Infectious allantoic fluid lyophilized, sealed under vacuum and stored in a refrigerator, has remained viable for at least 30 years (reviewed in 21). Attenuated vaccines are lyophilized in the presence of sucrose or lactose to preserve potency and extend shelf life.

pH Stability

In one survey, the reduction in titer following a pH 3 treatment at room temperature for 4 hours varied from 1–2 log10, for most isolates, to 5 log10 for others (reviewed in 21). Infectious bronchitis virus in cell culture was more stable in medium at pH 6.0 and 6.5 than at pH 7.0 to 8.0.

Chemical Agents

Infectious bronchitis virus is ether-labile, but some strains survived 20% ether at 4°C for 18 hours (reviewed in 21). All infectivity was destroyed by 50% chloroform at room temperature after 10 minutes and 0.1% sodium deoxycholate at 4°C for 18 hours. Infectious bronchitis virus is considered to be sensitive to the common disinfectants. Several have been compared for activity against another coronavirus, transmissible gastroenteritis virus of swine. Treatment with a final concentration of 0.05 or 0.1% beta-propiolactone (BPL) or 0.1% formalin eliminated IBV infectivity. Only the BPL treatment had no adverse effect on IBV hemagglutination antigen activity.

Strain Classification

Many methods are used to differentiate and classify isolates of IBV, and they have been thoroughly compared by de Wit (50). Serotype and more recently genotype classification, based on features of the S protein, are used to classify strains. Dozens of serotypes and genotypes of IBV have been detected, and many more will surely be reported in the future. Traditionally, IBV serotypes have been defined by VN and HI tests. Serotype-specific antibody is induced by the S1 subunit of the S protein. VN and HI are not commonly used for serotyping because of the limited availability of the increasing number of reference sera, corresponding to different serotypes, which are required for analysis. Some laboratories have used monoclonal antibodies that are specific to a given serotype, the antibodies corresponding to epitopes formed by the S1 protein. These monoclonal antibodies can be used in enzyme-linked immunosorbent assays (ELISAs), which are more economical than VN assays. However, serotype-specific monoclonal antibodies are only available for a small number of serotypes. Routinely now, laboratories are using reverse transcriptase polymerase chain reactions to produce DNA copies of IBV genes, usually the S1 part of the S protein gene, followed by sequencing or, less frequently, restriction endonuclease analysis. (See the "Confirmation of Infectious Bronchitis Virus by Nucleic Acid-Based Methods" section later in this chapter.) Such nucleic acid approaches then define IBV isolates by genotype rather than by serotype. Sequence analysis of field strains suggests that the evolution of IBV involves recombination during mixed infection (23, 92, 107, 112, 170). Consequently, IBV isolates with S genes of very similar sequence can vary substantially in other parts of the genome. The term "genotype" has no precise meaning and is defined operationally and arbitrarily by an author. That said, strains of a given serotype tend to have S1 amino acid identities of approximately 90% or greater; strains with this degree of sequence identity could be said to belong to the same S1 genotype. It is possible for isolates to be of the same S1 genotype and yet not of the same serotype or, at least, to cross-react poorly (24, 27). Sequence data of many isolates of IBV strongly supports the view that IBV evolution includes recombination. Probably all IBVs are recombinants, and probably at many positions within a given genome. However, it is not possible to state when recombination events occurred or which were the immediate parents of whichever part of the genome is being reported.

Serum Antibody Analysis

As stated previously, dozens of serotypes of IBV are known, defined by VN and HI tests. Virus neutralization has been performed with several systems (reviewed in 50). Cross-neutralization analysis of field isolates of Arkansas (Ark) and Delaware 072 has indicated the existence of subtypes of these serotypes (130, 137).

Strain classification by HI tests has also been investigated (reviewed in 50). The HI antibody response following a single exposure and resulting infection can be highly strain specific, even differentiating the Holland from the M41 strains of the same serotype (i.e., Massachusetts). The specificity of the early response and the limited cross reactivity are the basis for a procedure for serotyping isolates by HI. In contrast, Cook *et al.* (37) compared the HI test with the VN test in TOCs and concluded that the HI test was subject to high and variable cross-reactions and that IBV strains were more clearly differentiated by the VN test. Both studies based the evaluations on the results from primary sera, because secondary sera are much more broadly reactive (65). IBV must be treated with certain enzyme preparations, the active component of which is a neuraminidase (152), to generate HA activity (reviewed in 50, 64). The use of the HI and VN tests to characterize IBVs has declined as the application of RT-PCR and sequencing has increased.

Monoclonal Antibody Analysis

Monoclonal antibodies have been developed against several serotypes of IBV, although this is only a small proportion of the number of serotypes known to exist (85, 87, 88, 98, 101, 106). The VN epitopes, as defined by monoclonal antibody analysis, are situated largely in the first and third quarters of the S1 gly-

copolypeptide (24, 106). Antigenic groups of Australian isolates characterized by monoclonal antibodies correlated better with *in vivo* cross-protection data than groups defined by antisera (85). The use of monoclonal antibodies to identify isolates is discussed further in "Diagnosis."

Nucleic Acid Analysis

The sequence of the whole genome has been established for several isolates, and the sequence of the structural protein genes has been reported for a very large number of IBV strains. The gene that has been sequenced most frequently is that encoding the S1 subunit of the spike glycoprotein, as it is the S1 protein that determines serotype and, most importantly, is the major inducer of protective immunity. Moreover, the great sequence variation exhibited by the S1 protein (171) is believed to be largely responsible for strains of one serotype being able to replicate and cause disease, despite immune responses induced by another serotype. A great many S1 protein subunit gene sequences have been published and deposited in nucleotide sequence data banks, and gene sequences of some additional isolates have been published in data banks only. Comparison of the deduced S1 amino acid sequences has revealed that many of the serotypes defined by VN tests commonly differ by about 20–25%, and occasionally, by approximately 40% or more (68). Exceptions, however, exist. For example, the Connecticut 46 and Massachusetts 41 strains are in different serotypes, yet their S1 proteins differ by only 7.6% of amino acids (4.6% of nucleotides). Similarly, several isolates that had 97% amino acid identity with Dutch isolate D274 were defined in serum VN tests as belonging to different serotypes (24). These findings and the sequencing of VN-monoclonal-antibody escape mutants (98) suggest that only a few S1 epitopes induce the major VN antibodies and that a few key mutations in these epitopes might result in changes to a new serotype. Relationships established by more than one research group using VN tests do not always agree either with each other or with sequence analysis. For example, Johnson and Marquardt (93) considered that Arkansas 99 and Connecticut 46 were different serotypes, consistent with the finding that they differ by 29% in the first 200 S1 residues; whereas previously they had placed them in the same serotype. Experiments suggest that the degree of cross-protection between strains decreases as the differences between their S1 sequences increases (25, 68, 111).

Moderate sequence variation may be exhibited within a serotype. Thus, isolates of the North American Arkansas (Ark) serotype had S1 identities of ≥93% (nucleotide) and ≥89% (amino acid) among each other (137). Analysis of isolates of the 793/B (4/91; CR88) serotype from several countries isolated over a 15-year period indicated nucleotide and amino acid identities of ≥96% and ≥92%, respectively (15, 28).

Extensive sequence comparisons, from gene 2 (S) down to the 3′ end of the genome, suggest that recombination is a feature of IBV evolution (23, 92, 108, 170). Thus, it must not be assumed that if two isolates have very similar S proteins, deduced by whatever technique, that they are necessarily very similar in all other genes. Additional aspects of nucleic acid analysis are discussed in "Diagnosis."



4.2. Comparison of normal 16-day-old embryo (left) and curled, dwarfed, and infected embryo of the same age (right).

Laboratory Host Systems

Chicken Embryos

Most isolates of IBV replicate well in 10- to 11-day-old embryonating chicken eggs following inoculation of the allantoic cavity; field isolates often require adaptation via several (three or more) serial passages to achieve high titers of virus in the allantoic fluid (AF). Characteristic lesions such as stunting (dwarfing) and curling of the embryo and its feet (121) occur with increasing passage as does the incidence of embryo mortality. Upon opening the air cell end of the egg, the embryo is seen curled into a spherical form with feet deformed and compressed over the head and with the thickened amnion adhered to it (Fig. 4.2). A common internal lesion of the IB-infected embryo is the presence of urates in the mesonephros of the embryonic kidney. This lesion is not pathognomonic for IBV infection and is observed in embryos infected with avian adenovirus.

Embryo survival of 90% through the 19th day of incubation is characteristic of IBV field isolates upon primary isolation. In contrast, embryo mortality by the 10th passage may be as high as 80%. Characteristic embryo changes are seen several days after inoculation of the virus (121). Only slight movement of a dwarfed embryo may be observed during candling.

The optimum age of embryo inoculation, and the temperature and length of incubation for maximum infectivity titer of the Beau-

dette strain following allantoic cavity inoculation have been studied and reviewed (97). In general, inocula of about 103 tracheal organ culture infectious doses or 10^4 EID_{50} of IBV have yielded near maximum titers by 36–40 hours at 37°C. Microscopic lesions in embryos infected with IBV-M41 strain have been studied by Loomis *et al.* (121). Congestion with perivascular cuffing and some necrosis of the livers by the sixth day after inoculation was observed. Lungs were pneumonic, characterized by congestion, cellular infiltration, and serous exudate in the bronchial sacs. Interstitial nephritis with edema and distension of the proximal convoluted tubules and the presence of casts was noted in the kidney. Glomeruli were not altered. The chorio-allantoic membrane (CAM) and amniotic membrane were edematous. No inclusion bodies were observed. Coronaviruses isolated from pheasants have been propagated readily in chicken embryos.

Cell Cultures

Chicken kidney cells form syncytia, which quickly round up and detach from the culture surface, appear as large spheres with the refractile contents. Syncytia in Vero cells infected with Vero cell-adapted IBV-Beaudette contain numerous nuclei, and the cells remain attached to the culture surface longer.

The lag phase of IBV is 3–4 hours, with peak titers by 12 hours, although maximum titers in culture medium could be later (e.g., 24–30 hours, depending on the multiplicity of infection (16, 17, 78)). Titration of IBV in embryonating eggs gives higher titers (10- to 100-fold) than in CEK or CK cells. Infectious bronchitis virus strains, which had been passaged in embryos and many times in CK cells, replicated in chicken embryo fibroblast cultures, but to titers several log10 less than in CK cells (16, 139). The Beaudette, M41, and Iowa 97 strains have been propagated successfully in the African green monkey Vero cell line, which has been used for many fundamental studies of IBV Beaudette replication (116). Of 10 strains examined, two and none replicated in BHK-21 and HeLa cells, respectively, the titers in BHK cells being lower than in CK cells (16, 139). In summary, CK cells are widely used with many isolates of IBV, and only the Beaudette strain has been used extensively in Vero cells.

Pheasant coronavirus replicates well in embryonating chicken eggs (73), although not in chicken tracheal organ cultures. TCoV has not been grown successfully in cell cultures; turkey embryos are used (see Chapter 12).

Organ Cultures

The propagation of IBV in organ cultures of trachea and other tissues has been reviewed by Darbyshire (47). Tracheal rings are prepared from 20-day-old embryos and maintained singly in roller tubes. Following infection with IBV, ciliostasis, which is easily observed by low-power microscopy, occurs within 3–4 days, earlier with high-titering inocula. Tracheal organ cultures have proved very useful for the isolation, titration, and serotyping of IBV, because no adaptation of field strains is required for growth and the induction of ciliostasis. However, some strains with affinity for other tissues do not cause consistent ciliostasis. When titrated in tracheal rings, preparations of the Belgian B1648 nephropathogenic strain gave the appearance of being of low titer, whereas titration in embryonating eggs revealed the titer to be high (D. Cavanagh, unpublished observation). Pheasant coronavirus replicates to only very low titer in chicken tracheal organ cultures.

Pathogenicity

Infectious bronchitis is primarily a disease of chickens. Infection is initiated via the respiratory tract regardless of the tissue tropism of the strain (respiratory, kidney, gonad). The virus replicates and produces lesions in many types of epithelial cells, including those of the respiratory tract (nasal turbinates, Harderian gland, trachea, lungs, and air sacs), kidney, and gonads (oviduct, testes) (11). The virus also grows in many cells of the alimentary tract (esophagus, proventriculus, duodenum, jejunum, bursa of Fabricius, cecal tonsils, rectum, and cloaca) many times with little pathobiological clinical effect (6, 100, reviewed in 19). Some Asian strains may cause lesions in the proventriculus. The virus commonly persists in the alimentary tract in young chickens (5) and in layers in the absence of clinical disease (96). Some live vaccine strains such as the Arkansas in the United States have been shown to persist in the respiratory tract especially when administered in combination with another attenuated strain (e.g., Massachusetts) (5).

Infectious bronchitis virus strains damage the respiratory epithelium often predisposing young chickens to secondary infections with pathogenic bacteria. Airsacculitis and systemic colibacillosis are common outcomes of virulent IBV infection especially in broiler chickens (127). Laboratory inoculation with M41 followed by aerosol administration of an 02 or 078 strain of *E. coli* produced disease often observed under commercial conditions. An interval of 4 days between IBV and *E. coli* challenges produced airsacculitis, pericarditis, and perihepatitis. Administration of IBV alone did not induce lesions (142). Experimentally, chickens inoculated with IBV and *E. coli* had more severe and persistent respiratory lesions than those inoculated with IBV alone. *E. coli* was isolated from the tracheas of chickens inoculated with IBV and *E. coli* more frequently than from chickens inoculated with IBV alone. There was neither increased numbers of *E. coli* nor significant lesions in the respiratory tract of the group inoculated with *E. coli* alone (133). Laboratory studies demonstrated ascites in 15% of broilers infected with M41 followed four days later by *E. coli* innoculation (166).

Infection with IBV has long been recognized to enhance disease and shedding caused by pathogenic avian mycoplasma. *M. gallisepticum* and *M. imitans* also exacerbate the consequences of infection by IBV (reviewed in 61). Interaction between combinations of Newcastle disease and infectious bronchitis live vaccine, *M. gallisepticum* and *E. coli* resulted in profuse multiplication of *E. coli* with severe and persisent histological lesions and mortality in SPF chickens inoculated at one-week of age (134). Virulent IBV given three days prior to an aerosol exposure to *Mycoplasma synoviae* was shown to increase the incidence of arthritis in layer pullets. Strain M41 induced a higher incidence than D1466 in birds given *M. synoviae* (114). Another report showed the pathogenic M41 strain of IBV rapidly activated an apparent latent *M. gallisepticum* in the trachea induced by oral

fluoroquinolone treatment of SPF chickens (148). In a case-controlled study of pheasants, sinusitis was commonly observed and was associated with *M. gallisepticum* in the trachea and conjunctiva. Sinusitis was also attributed to the presence of pasteurella and avian coronaviruses, the most common infectious agents detected (174).

Immunosuppression increases the severity of respiratory disease associated IBV–*E. coli* combined infections and produces significant economic losses (136). Commercial broilers infected with infectious bursal disease virus (IBDV) are more susceptible to develop secondary infection of the respiratory tract with *E. coli*, resulting in significant economic losses. Phagocytosis of *E. coli* by macrophage was markedly reduced in chickens infected with IBDV and IBV. IBDV immunosuppression prolonged IBV shedding (150).

Vaccination for IBV and lentogenic field strains of Newcastle disease virus has been used to control airsacculitis in broilers. Vaccination using the Massachusetts serotype H 120 vaccine at one day of age prevented broilers from developing clinical IB and significantly reduced *E. coli* airsacculitis after challenge with M41 and *E. coli* strain 506 (127).

Nephropathogenic strains may not produce significant respiratory lesions (72) or clinical signs (179). Field isolates, regardless of originating tissue, infect via the respiratory tract and produce lesions of varying severity in the trachea depending on their virulence.

Cumming (46) enumerated some of the management factors that contribute to IB-related kidney disease in Australia. Greater mortality was seen in males, where there was cold stress, in certain breeds, and/or where animal by-products were the major component of high-protein diets. Some of these factors known to exacerbate the clinical disease have been used in experimental models to evaluate the clinical outcome of interaction between such factors and different IBV strains. Chickens fed increased levels of dietary calcium followed by infection with the nephropathogenic Gray IBV strain frequently developed urolithiasis and kidney lesions, but a similar infection introduced 8 weeks prior to feeding chickens a diet including increased levels of calcium did not induce urolithiasis (72).

Other differences in virulence of IBV strains have been noted. Passage of IBV in chicken embryos gradually results in a decrease in virulence for chickens; this is the traditional way of developing IB vaccines. At least some attenuated strains cause little or no ciliostasis after application to chickens by eye drop and nasal instillation, although they do cause ciliostasis following inoculation of tracheal organ cultures (60, 77). As attenuated virus may replicate to lower titers in the nose than does virulent virus (77), the maintenance of ciliary activity in the trachea may be at least in part because the initial replication in the nares produces only low amounts of virus, which have little subsequent effect on the trachea. Although some strains of IBV are highly nephropathogenic, causing extensive and reproducible kidney disease in experimental conditions, many strains of IBV may be associated with nephritis to some degree in the field; environmental factors are probably important as to whether kidney complications are significant. Tissue tropism of a Connecticut serotype strain of IBV could be altered from respiratory to kidney tissues by *in vivo* serial passage of the virus via the cloacal route (168). Following 13 such passages, the virus grew better in kidney tissues and caused more obvious lesions there than did the parent virus. The kidney and other non-respiratory organs are sites of persistence of IBV infectious virus being periodically shed in nasal secretions and feces (55,63).

Virulence for the reproductive tract may also differ among IBV strains. Presence of maternal antibody could prevent damage to the oviduct during an early-age IBV infection (32). In susceptible layers, different IBV strains produced a range of effects varying from shell pigment changes with no production drop to production drops of 10–50% (82). From China (178), outbreaks of a "proventricular-type" IBV infection in broiler chickens have been reported since 1996. The disease is characterized by swollen and hemorrhagic ulceration of proventriculi and mortality ranging from 15–80%. Also reported in China (180) have been chicks showing depression, swollen eyes with lacrimation, and diarrhea followed by obvious signs of respiratory disease. The morbidity of this disease was 100%, and the mortality was 20%. Postmortem examination showed that the primary lesion was swelling of glandular stomach. IBV was isolated, one isolate being named QX IBV.

Coronaviruses isolated from pheasants differ in gene sequence from IBV to an extent similar to that exhibited by different serotypes of IBV (24). When three isolates of coronavirus from pheasants were inoculated into chickens, no signs of disease were observed (118), leading to pheasant coronavirus (PhCoV) being officially considered as a species distinct from IBV. PhCoV is associated with both respiratory and kidney disease in pheasants (4, 27, 73, 118, 143). Turkey coronavirus, which has not been observed to cause disease in chickens following experimental inoculation, is associated with enteric disease in turkeys (see Chapter 13). The H120 vaccinal strain of IBV has been isolated from peafowl (Pavo cristatus) in China, and a nephropathogenic (in chickens) strain of IBV has been isolated from a teal (Anas sp.) (119). In both cases these birds were being raised domestically in the vicinity of chickens. In neither case was disease reported in the peafowl or teal. A coronavirus with all gene sequences having high identity with IBVs was isolated from a partridge in China. In Brazil a coronavirus having antigenic identity with IBV has been isolated from domestic guinea fowl, in the proximity of commercial chickens that were exhibiting mortality, enteritis, and low feed consumption. The virus caused respiratory distress and watery feces in both chickens and guinea fowl following experimental inoculation. Whether the guinea fowl virus was a *genuine* guinea fowl virus or an IBV that had transferred to the guinea fowl from chickens is not known. The point is perhaps moot. Taken together, the preceding observations indicate that IBV can replicate in avian species other than chickens, indeed in birds that are not gallinaceous, and that coronaviruses of other birds can replicate in chickens, although not necessarily causing disease. Coronaviruses have been detected in graylag goose, mallards, and pigeons. Limited sequencing suggests that these viruses should be assigned to Group 3, like IBV, but has also shown that these viruses were clearly not very closely related to IBV (e.g., they had one or two additional genes located after the

N protein gene (20, 95)). A coronavirus has also been isolated from a green-cheeked Amazon parrot (*Amazon viridigenalis Cassin*), though, on the basis of limited sequence data, this would appear to be substantially different from IBV (74) and possibly not a Group 3 coronavirus. A Group 2 coronavirus has been isolated from a Manx sheerwater (*Puffinus puffinus*) (138).

Pathogenesis and Epizootiology

Natural and Experimental Hosts

As explained previously, it is no longer considered that the chicken is the only host for IBV, although it is possible that it is only in the chicken that IBV would cause disease.

Age of Host Commonly Affected

All ages are susceptible, but the disease is most severe in baby chicks, causing some mortality. As age increases, chickens become more resistant to the nephropathogenic effects, oviduct lesions, and mortality due to infection (2,44, 157).

Transmission, Carriers, and Vectors

Infectious bronchitis virus spreads rapidly among chickens in a flock. The disease is highly contagious and has a very short incubation period. Susceptible birds placed with infected chickens usually develop clinical signs within 24–48 hours. Virus was isolated consistently from the trachea, lungs, kidney, and bursa of chickens at 24 hours and through the seventh day after aerosol exposure (80). The frequency of virus isolations declined with time and varied with the infecting strain, but IBV was isolated from the cecal tonsils at 14 weeks and from feces 20 weeks post infection (3). Re-excretion of IBV has also been detected from hens that had been virus-negative for several weeks following recovery from inoculation at one day of age. Virus was isolated from tracheal and cloacal swabs collected at the point of lay and 19 weeks of age (96). The nature of the persistence of IBV infection remains undefined, although the kidney may be one of the sites of persistent infection (55). IBV vaccine virus may persist in various internal organs for up to 163 days or longer (63). During this period, the virus may be periodically shed in nasal excretions and feces. Reports of extended and intermittent shedding are evidence of the potential risk of flock-to-flock transmission via contamination of personnel or equipment.

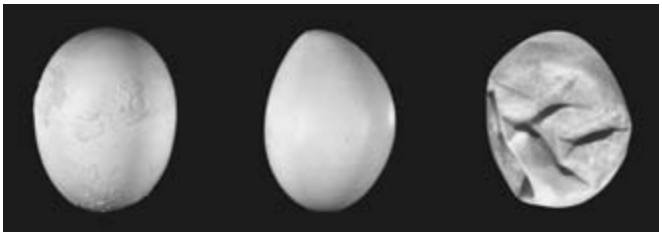
The frequency of airborne spread between flocks is unknown, although it is generally considered that IBV spreads readily. In view of the recent discovery of IBV in species other than the chicken, it should be considered that some other species of bird may act as vectors of IBV.

Incubation Period

The incubation period of IB is dose dependent and is as short as 18 hours for intratracheal inoculation and 36 hours for ocular application.

Clinical Signs

The characteristic respiratory signs of IB in chicks are gasping, coughing, sneezing, tracheal rales, and nasal discharge. Wet eyes may be observed, and an occasional chick may have swollen sinuses. The chicks appear depressed and may be seen huddled under a heat source. Feed consumption and weight gain are significantly reduced. In chickens greater than 6 weeks of age and in adult birds, the signs are similar to those in chicks, but nasal discharge does not occur as frequently, and the disease may go unnoticed unless the flock is examined carefully by handling the birds or listening to them at night when the birds are normally quiet.



4.3. Thin-shelled, rough, and misshapen eggs laid by hens during an outbreak of IB. (Van Roekel)

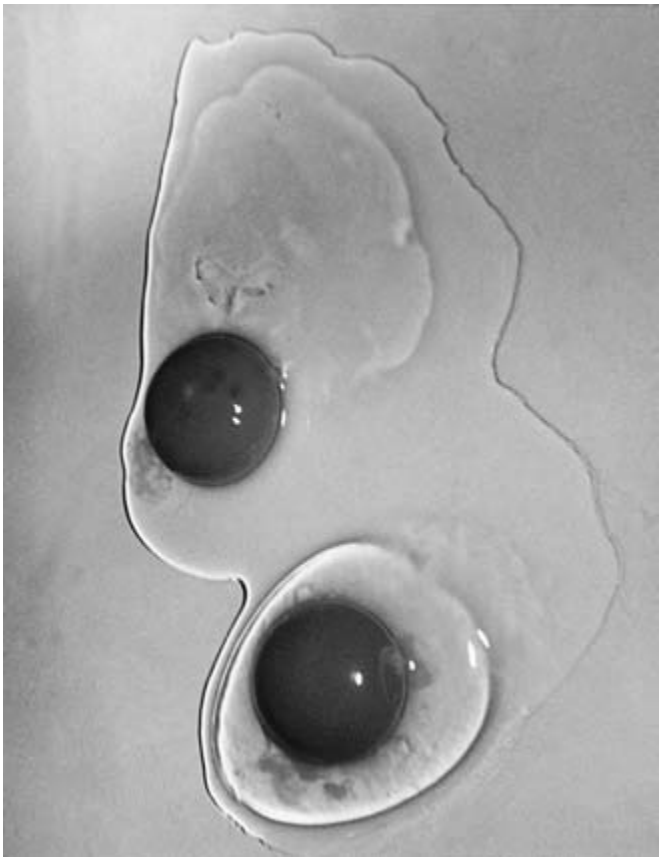
Broiler chickens infected with one of the nephropathogenic viruses may appear to recover from the respiratory phase and then show signs of depression, ruffled feathers, wet droppings, increased water intake and mortality (46, 175). When urolithiasis is associated with IB in layer flocks, there may be increased mortality, but otherwise the flock appears healthy (13, 42).

In laying flocks, declines in egg production and quality are seen in addition to respiratory signs. Infectious bronchitis virus, however, has been isolated from cloacal swabs or cecal tonsil samples from breeder or layer flocks with slight production drops and the production of pale unpigmented shell eggs, but no respiratory signs. The severity of the production declines may vary with the period of lay (57) and with the causative virus strain (82). Six-to-eight weeks may elapse before production returns to the pre-infection level, but in some cases, this is never attained. In addition to production declines, the number of eggs unacceptable for setting is increased; hatchability is reduced; and soft-shelled, misshapen, and rough-shelled eggs are produced (43) (Fig. 4.3).

Internal quality of eggs, as observed when breaking eggs on a flat surface, may be inferior. The albumen may be thin and watery without definite demarcation between the thick and thin albumen of the normal fresh egg. (Fig. 4.4).

Infectious bronchitis virus infection of one-day-old chicks can produce permanent damage to oviducts leading to reduced egg production and inferior quality eggs when the chickens come into lay. The severity of oviduct lesions is likely to be less in infections of older chickens, and some serotypes may fail to produce any pathologic change even in infections of one-day-old chicks. Presence of specific maternal antibody was also shown to protect oviduct from damage due to IBV infection in early life (32).

Coronavirus infection of laying pheasants has been associated with poor hatchability, small size, and variable color—pale buff to greenish brown, compared to the more normal dark olive brown



4.4. Contents of two eggs. Normal egg (bottom). Egg from chicken exposed to IBV at one day of age (top). Note watery albumen with yolk separated from thick albumen. (Hofstad)



4.5. Kidney lesions associated with IB caused by T strain of virus. Note swollen kidneys with tubules and ureters distended with urates. (Cumming)

(118). On another game farm from which a coronavirus was isolated, 15% of breeding pheasants died rapidly, the only clinical sign being sneezing. On that occasion, egg production and hatchability were down, but egg quality was unaffected. In a group of pheasant poults, no respiratory signs were reported, although by 10 weeks of age mortality had reached 45%. The sick birds were reported as having ruffled feathers, with drooping wings (118).

Morbidity and Mortality

All birds in the flock become infected, but mortality is variable depending on virulence of the infecting serotype; age; status of immunity, either maternal or active; and stresses such as cold or secondary bacterial infections. Moderate to severe mortality has been noted with some of the respiratory and nephropathogenic strains, such as Delaware 072 and Australian T strain, respectively. Sex, breed, and nutrition are additional factors that contribute to the severity of kidney disease (45, 46). Mortality may be as high as 25% or more in chickens less than 6 weeks of age and usually is negligible in chickens greater than 6 weeks. Mortality in urolithiasis cases ranged from 0.5–1.0% per week.

Mortality of 45% by 10 weeks of age has been reported on a pheasant game farm, from which a coronavirus was isolated (118). Mortality can also occur in adult pheasants; 15% died on one farm.

Gross Lesions

Infected chickens have serous, catarrhal, or caseous exudate in the trachea, nasal passages, and sinuses. Air sacs may be foamy during the acute infection (Fig. 4.6 A), then become cloudy and contain a yellow caseous exudate. Areas of pneumonia may be observed around the large bronchi. Nephropathogenic infections produce swollen and pale kidneys with the tubules and ureters often distended with urates (45, 179) (Fig. 4.5).

Fluid yolk material may be found in the abdominal cavity of chickens that are in production, but this is also seen with other diseases that cause a marked drop in egg production. Permanent lesions in the oviduct may be a consequence of IBV infection of one-day-old chicks and are a cause of reduced egg production when reaching maturity. The middle third of the oviduct is most severely affected and may be nonpatent and hypoglandular. In addition, effects of IBV infection on the reproductive tract of chickens in production have been detailed by 155. They observed reduced length and weight of the oviduct in infected birds as well as regression of the ovaries.

The most noted lesions associated with the infection of pheasants by coronaviruses in the field are visceral urate deposition ("visceral gout") and urolithiasis with gross swelling of the kidneys, which are pale (118, 143).

Histopathology

The mucosa of the trachea of chickens with IB is edematous. There is a loss of cilia, rounding and sloughing of epithelial cells, and minor infiltration of heterophils and lymphocytes within 18 hours of infection. Regeneration of the epithelium starts within 48 hours. Hyperplasia is followed by massive infiltration of the lamina propria by lymphoid cells and the formation of a large number of germinal centers, which may be present after seven days. If air sac involvement occurs, there is edema, epithelial cell desquamation, and some fibrinous exudate within 24 hours. Increased heterophils can be observed later with lymphoid nodules, fibroblast proliferation, and regeneration by cuboidal epithelial cells (149) (Fig. 4.6 A–F).

Intraocular inoculation of H120 vaccine virus resulted in the replication of the virus in the Harderian gland (as well as in respiratory tissues) with histologic changes characterized by the presence of Russell bodies in plasma cells and the exfoliation of tubular epithelial cells (165).

The kidney lesions of IB are principally those of an interstitial nephritis (Fig. 4.6 G–H). The virus causes granular degeneration, vacuolation and desquamation of the tubular epithelium, and massive infiltration of heterophils in the interstitium in acute stages of the disease. The lesions in tubules are most prominent in the medulla. Focal areas of necrosis may be seen as well as indications of attempted regeneration of the tubular epithelium. During recovery, the inflammatory cell population changes to lymphocytes and plasma cells. In some cases, degenerative changes may persist and result in severe atrophy of one or all of the divisions of the nephrons. In urolithiasis, the ureters associated with atrophied kidneys are distended with urates and often contain large calculi composed mainly of urates (149).

Ultrastructural studies of infected kidney tissues revealed epithelial cells of the lower nephron and ducts to be the primary targets for IBV replication (29, 30). Infected epithelial cells containing virus particles were more abundantly found in the collecting ducts, collecting tubules, distal convoluted tubules, and Henle's loops than in the proximal convoluted tubules. Cytoplasmic changes in the infected epithelial cells were characterized by swelling of the mitochondria, dilation of Golgi vesicles, and an increase in the amount of rough endoplasmic reticulum (RER). Virus particles were seen budding from the RER, and as the virus replication progressed, virus particles were enclosed in dilated RER, cytoplasmic vesicles, or virus containing electron-dense bodies.

Experimental IB infection of the oviduct of mature hens resulted in decreased height and loss of cilia from epithelial cells; dilation of the tubular glands; infiltration by lymphocytes, other mononuclear cells, plasma cells, and heterophils; and edema and fibroplasia of the mucosa of all regions of the oviduct (149, 155). The histopathology of IB and comparisons with other diseases are given in detail in a book by Riddell (149), a review of renal pathology by Siller (156), and more recent investigations (29, 30, 179).

Histopathological analysis of pheasant poults infected with a coronavirus revealed moderately severe interstitial nephritis. There was tubular infiltration with predominantly mononuclear cells, tubule dilation, flattening of lining of epithelium, cast formation, and focal necrosis (118, 143).

Immunity

Active

Aspects of immunity to IBV have been reviewed previously (19, 55). Both breed- and strain-related genetic resistance to IBV infection have been described in chickens (8, 14, 39, 141, 157). More investigations, however, are warranted to assess the genetic resistance of commercial lines of chickens. Chickens just recovered from the natural disease are resistant to challenge with the same virus (homologous protection), but the extent of protection to challenge with other IBV strains (heterologous protection) varies. Factors that complicate studies of the mechanism and duration of immunity to IB are the multiple serotypes that are recognized (see "Incidence and Distribution"), the variation in virulence observed among strains (see "Pathogenicity"), and the different manifestations of IBV infection for which protection may be needed (see "Clinical Signs").

Challenge of vaccinated birds with homologous virus results in much lower titers of recovered challenge virus, and for a shorter period, than in unvaccinated birds (36, 53, 113, 144). When the challenge virus is of a heterologous type, the challenge virus may replicate to high titers and cause clinical disease. Experiments with recombinant IBV having a heterologous S gene have confirmed that it is differences in the S protein that determine poor cross-protection (77).

Respiratory protection is usually evaluated 3–4 weeks after an IB infection or immunization and has been done in several different ways. Challenge routes include tracheal, intranasal, and by eyedrop. The failure to recover IBV from the trachea at 4–5 days post challenge has been used as a single criterion of immunity (79). More comprehensive evaluations have included two or more additional criteria of resistance to challenge, including the failure to isolate virus from the kidney and oviduct, no clinical signs of IB, no tracheal lesions, or the presence of tracheal ciliary activity (11, 48, 77, 176). Accumulating scores from the different criteria are used to indicate the range of protection from full to partial or none. An alternative approach is an evaluation of vaccinated chickens for protection against mortality from a challenge with a mixture of IBV and *E. coli*. This method showed evidence of more vaccinal cross-protection than found with other assessments of tracheal immunity (36).

Protection against mortality from nephritis is important as evidence of satisfactory vaccinal immunity in which nephritis is a major clinical problem (104, 147). The ability to reduce or prevent egg-production declines from a challenge infection is evidence of IB protection in a laying flock (12).

Although it is established that the S1 subunit of the S protein induces VN and HI antibodies and protective immunity (86, 94, 124, 158), knowledge of the mechanism of protection against

clinical disease is incomplete. Local respiratory tissue immune mechanisms are important in protection. However, the role of local antibody in preventing reinfection is unclear. Some studies have reported neutralizing antibody in nasal secretions to play a role in preventing re-infection (81) and that the Harderian gland contributes to local immunity (49). The protective role of antibody is also evident by the fact that chickens immunocompromised by IBDV infection suffer more severe episodes of IBV infection than their immunocompetent counterparts (150, 161). IBV antibody was detected in tears of vaccinated chickens both by ELISA and virus neutralization assay (66). However, antibody levels in tears were not accurate indicators of IBV immunity as determined by respiratory challenge by IBV. Antibody does not appear to be the only source of resistance, however, as demonstrated in chickens treated with cyclophosphamide or bursectomized in ovo and then exposed to IBV (33, 38). In those trials, no antibody could be detected, but the chickens resisted IBV challenge. Evidence of cell-mediated immune responses to IBV (60, 140) are lymphocyte transformation assays of live and inactivated virus vaccinates (163), cytotoxic lymphocyte activity (34, 153, 154), delayed type hypersensitivity (35), natural killer cell activity (160), and histologic evidence for significant T-cell (especially CD4+ phenotype) infiltration in the respiratory and kidney tissues of IBV-infected chickens (90). Cytotoxic T lymphocyte responses peaked at 10 days after IBV infection and correlated well with the decline of IBV in lungs and kidney (163). IBV-specific CTL epitopes were mapped to within the carboxy-terminal 120 amino acids of the nucleocapsid protein (164).

Passive

Maternally-derived antibody (MDA) can reduce both the severity of vaccinal reaction and the efficacy of the vaccine if the vaccine is of the same type used in the breeder flock immunization (104, 105). Despite this, vaccination of maternally immune one-day-old commercial chicks is routinely performed. In one study, MDA provided protection against challenge at one day and one week but not at two weeks of age (129); whereas in another study, MDA did not reduce the titer of re-isolation of challenge virus given at two days of age (177). Mondal and Naqi (130) showed that chicks hatched with high levels of MDA had excellent protection (>95%, assessed by non-recovery of challenge virus) against challenge at one day of age but not at seven days (<30%). This protection significantly correlated with levels of local respiratory antibody and not with serum antibody. A high percentage of both MDA+ and MDA- chicks failed to produce IBV antibody when vaccinated at one day of age by the intraocular route. In addition, MDA+ chickens had a weaker virus-neutralizing antibody response to a second IBV vaccination compared to MDA- birds. MDA+ chicks experienced a more rapid decline in MDA after one-day-of-age vaccination compared to their unvaccinated counterparts.

Diagnosis

Diagnosis of IB is based on the clinical history, lesions, seroconversion or rising IBV antibody titers, IBV antigen detection by a number of antibody-based antigen capture assays (described later), virus isolation, and, increasingly, by detection of IBV RNA. Diagnosis of IB should include, if possible, identification of the serotype or genotype of the virus because of the great antigenic variation exhibited by IBV strains and the availability of vaccines designed for different serotypes. The many approaches to the detection of IB virus or antibodies induced by it have been described and critically compared by de Wit (50). At the outset, it should be stated that no technique, whether based on antibodies or nucleic acid technology, is totally satisfactory for confirmation of infection by a specific IBV serotype in the field.

Isolation and Identification of the Causative Agent

Although primarily a respiratory pathogen, IBV can grow in non-respiratory (kidney, oviduct, alimentary tract) tissues. Knowledge of the pathogenesis of IBV, reviewed by Dhinaker Raj and Jones (55), has been instructive for the detection of the virus. Factors to be taken into account include the time elapsed between infection and sampling and the immune status of the bird at infection. These and other factors have been discussed by de Wit (50).

Virus Isolation

The trachea is a primary target for IBV and is, therefore, a preferred sampling site, especially within the first week of infection. The sample could be either tracheal swabs or tracheal tissue collected at post mortem. In individual chickens, titers of IBV are maximal by day four or five after which they decline rapidly. Cloacal swabs or cecal tonsils collected during post mortem examination, however, can be of particular value in cases in which more than one week may have elapsed since the start of infection. This is in part because the virus grows initially in the upper respiratory tract and spreads to non-respiratory organs. Consequently, the virus generally is cleared from the trachea sooner than from the intestinal tissues. Additionally, evidence exists that IBV can persist, especially in non-respiratory tissues e.g., kidney (3, 96, 122). Samples from the lung, kidney, and oviduct should also be considered depending on the clinical history of the disease. Sample selection from extremely large flocks can be a difficult problem. The placement of susceptible sentinel chickens in a problem flock has been successful when direct sampling methods in the flock had failed (67). Sentinels are removed for direct sampling after one week of contact exposure. Procedures for sample collection and processing for IBV isolation have been described in detail (50, 67).

Samples for virus isolation commonly are inoculated into embryonating chicken eggs or tracheal organ cultures preferably from a specific-pathogen-free source. Fluids should be harvested after 48–72 hours from either culture system for blind passage into another set of cultures. Samples should receive at least 3–4 blind passages before being called negative based on failure to cause death or lesions in embryos. Ciliostasis may be observed upon initial passage in tracheal organ cultures. These observations are not in themselves sufficient to confirm the presence of IBV; the virus presence must be confirmed by serological meth-

ods (e.g., VN, HI, ELISA), immunohistochemistry, nucleic acid analysis, or by electron microscopy. Antiserum collected at four weeks postinoculation should be suitable for use in two-way comparisons by VN test to determine the serotype of the isolate (50, 64).

Coronaviruses in pheasants have been detected by sampling respiratory tissues and kidneys (118) and by oral swabs collected when respiratory signs were evident (27). The viruses could be grown in chicken eggs after allantoic sac inoculation (118). In contrast, coronaviruses detected by RT-PCR in graylag goose, mallards, and pigeons could not be propagated in chicken eggs following allantoic sac inoculation (95).

Confirmation of Infectious Bronchitis Virus by Antibody-Based Methods

Detection of IBV directly using postmortem material may be attempted by a number of methods (reviewed in 50; 64). Sections or scrapings of the trachea mucosa and other tissues taken from birds at postmortem can be examined by immunofluorescence or immunoperoxidase assays, using IBV-specific polyclonal sera or monoclonal antibodies (76). The results are not always easy to interpret, especially from field specimens because of nonspecific reactions. Tracheal material can also be used in agar gel precipitin tests (AGPT), which has a sensitivity greater than might be expected (50). Bhattecharjee *et al.* (9) infected tracheal organ cultures with IBV and revealed the presence of the virus by immunofluorescence without fixation of the cultures using low power microscopy.

If virus propagation in embryonating eggs is attempted, then confirmation of the presence of IBV can be made by the AGPT test. Because the amount of IBV precipitin antigen in infected chicken embryos can sometimes be low, however, sections of the chorioallantoic membrane or cells sedimented from the allantoic fluid can be used for immunofluorescence or immunoperoxidase assays. Infectious bronchitis virus in allantoic fluid, or after growth in tracheal organ cultures, may also be detected and identified as to serotype, using monoclonal antibodies in indirect or antigen-capture enzyme-linked immunosorbent assay (ELISA) (reviewed in 50; 85, 88, 93, 122, 35).

Coronaviruses isolated from pheasants have, to date, been poorly cross-reactive in HI and VN tests using sera raised against IBV serotypes. The use of immunofluoresence or antigen-capture ELISAs, in conjunction with IBV antibodies, has not been attempted with coronaviruses from pheasants. Nucleic acid-based approaches have been successful extensively for demonstrating the presence of coronaviruses in pheasants. Antigenic cross-reaction between turkey coronaviruses and IBV has been demonstrated (120), and ELISAs have been used to detect coronavirus antibodies in turkey sera (75, 120, 165, 173).

Confirmation of Infectious Bronchitis Virus by Nucleic Acid-Based Methods

RT-PCRs have been applied directly to material from infected chickens or after preliminary amplification of the virus in embryonating eggs. Well-chosen RT-PCRs are sufficiently sensitive to detect IBV RNA extracted from mouth, tracheal swabs, or cloacal swabs (i.e., it is not essential to first isolate/amplify the virus in embryos). That said, because of the ubiquitous use of live IB vaccines, simply getting a positive PCR result is not sufficient for diagnosis; sequence of the PCR product should be compared with the corresponding sequences of vaccine strains.

RT-PCR genotyping methods have largely replaced HI and VN serotyping for determining the identity of a field strain. The molecular basis of antigenic variation has been investigated, usually by nucleotide sequencing of the gene coding for the spike (S) protein or, more specifically, nucleotide sequencing of the gene coding for the S1 subunit of the S protein in which most of the epitopes to which neutralizing antibodies bind are found (98, 106). An exact correlation with HI or VN results has not been seen, in that while different serotypes generally have large differences (20–50%) in the deduced amino acid sequences of the S1 subunit, other viruses that are clearly distinguishable in neutralization tests show only 2–3% differences in amino acid sequences (24). However, there is, in general, good agreement between data represented by the S1 sequence and the VN serotype. It is important to note that it is not possible at the present time to differentiate vaccine and pathogenic field strains on the basis of sequencing the S gene.

Advantages of genotyping methods include a rapid turnaround time and, importantly compared to VN and HI tests, the ability to detect a wide variety of genotypes, depending on the tests used. Clinical samples such as swabbings from the trachea or cloaca may be directly tested provided sufficient titer of the virus is present. Passage in embryonating eggs may be necessary to increase viral titer prior to RT-PCR analysis.

Restriction fragment length polymorphism (RFLP) RT-PCR differentiates IBV genotypes based on unique electrophoresis banding patterns of restriction enzyme-digested fragments of S1 following amplification of the gene by RT-PCR (5, 125).

S1 genotype-specific RT-PCR may be used to identify field isolates as IBV genotypes using universal primers and their specific genotypes using strain or type specific primers (102). S1 gene primers specific for genotypes Massachusetts (Mass), Connecticut, Arkansas, JMK, DE/072/92 and California have been developed (102).

Both the RFLP RT-PCR and S1 genotype-specific methods have limitations, however. Unknown variant serotypes may be detected as IBV using universal primers, but the specific serotype cannot be identified until the strain is sequenced and specific primers are designed.

Nucleotide sequencing of the S1 gene is the most useful technique for the differentiation of IBV and is the genotyping method of choice in many laboratories. RT-PCR product cycle sequencing of the hypervariable amino terminus region of S1 may be used diagnostically to identify previously unrecognized field isolates and variants (60, 103, 115, 145). Comparison and analysis of sequences of unknown field isolates and variants with reference strains for establishing potential relatedness are significant advantages of sequencing.

Dot blot hybridization has been reported as a simpler alternative to sequencing and RFLP technology that may not be available in some countries. Slot blot hybridization using RT-PCR

template DNA of the hypervariable regions of S1 was immobilized onto nitrocellulose membrane used to identify and characterize IBV strains. Digoxigenin-labeled probes from reference and unknown field viruses were synthesized and hybridized to template DNA. All reference strains could be distinguished and isolates could be identified by genotype if they were at least 95% identical to a reference strain (131).

The major uses of RT-PCR tests pertaining to the S gene are virus identification and its application in the understanding of epidemiology during IBV outbreak investigations. Similarities based on sequencing of S gene have also been shown to be a better predictor of cross-challenge results than traditional serotyping by VN (111). Genetic tests based on RT-PCR of the S gene do not provide information defining viral pathogenicity.

When only small amounts of viral RNA have been available (e.g., on swabs), nested PCRs, in which some of the DNA produced in the first PCR is used in a second PCR using two further oligonucleotides, have been used. However, nested PCRs are arguably too sensitive for routine diagnostic use. On the one hand, one might amplify low levels of residual vaccine virus, that would be misleading. On the other, tiny amounts of contaminating DNA may be amplified, giving a false positive.

Two strategies would seem to be worthy of further investigation. One is to do a single RT-PCR on RNA extracted from trachea or from swabs, using as much RNA as possible, using all the RT product in the PCR, and generating relatively small DNA products (e.g., approximately 600 bp or fewer). The genotype of IBV can be determined either by use of genotype-specific oligonucleotides or by sequencing the product. If sequencing is to be used, then "universal" oligonucleotides can be used in the RT-PCR; that is, oligonucleotides designed to work with many, if not all, types of IBV (1, 26, 102, 103). In that way, one is less likely to miss new types of IBV compared with the use of selected genotype-specific oligonucleotides.

The second approach is to first amplify the virus, from tissue or swabs, in embryonating eggs. This approach has been used extensively by Jackwood and colleagues (89) and Gelb and colleagues (68, 102, 103, 137). The growth of IBV in eggs prior to RT-PCR is preferable to the use of RNA extracted directly from swabs when relatively large (approximately 2000 bp) PCR products are required (109).

The high degree of similarity of the genomes of coronaviruses from turkeys and pheasants compared with IBV means that these viruses may be detected using some of the universal oligonucleotides designed for IBV (26).

Serology

The multiple IBV serotypes and the antigenic variation noted within the described types add complexity to the selection of an appropriate serologic method and the analysis of test results. All IBV serotypes would seem to have common epitopes (group-specific antigens), which is not surprising given the moderately high amino acid sequence identity within the N and M proteins and the S2 part of the spike protein. IBVs also induce type-specific antibodies, of course, determined by epitopes of the S1 protein.

The ELISA, immunofluorescence, and immunodiffusion tests bind antibody to group- as well as to type-specific antigens, hence these tests cannot differentiate serotypes. Following a first infection with IBV, most of the VN and HI antibody response is serotype-specific. A second infection, even with the same serotype, results in a more broadly reactive serum. As chickens in the field will almost certainly have been vaccinated against IB, and possibly infected more than once by field virus, field sera are not very useful for serotyping. Thus, single infection sera, raised experimentally using SPF chickens, are used for serotyping.

Routine serology is usually done with VN, HI, or ELISA tests, reviewed by de Wit (50). The group-specific AGPT can be used, but precipitating antibodies are short-lived, and the test may lead to under detection. Positive AGPT results are indicative of recent infection. De Wit *et al.* (51) vaccinated one-day-old broiler chicks with maternally derived antibody and nine-week-old SPF chicks with the H120 vaccine strain; neither group of birds gave antibody detectable by the AGPT. After challenge the AGPT was positive, sensitivity being about 40%. Infection with a virus of the same serotype as the vaccine may lead to poor production of antibodies, as detected by the AGPT (51, 52). Overall, the AGPT is not recommended for detection of IBV antibodies, being better used for detection of IBV antigen.

IBV ELISAs are group-specific (51, 99). The method is used widely, and kits for conducting the procedure are commercially available. ELISAs first detects IBV antibodies within one week of infection, earlier than by HI or VN tests (51, 52, 126, 128). Two serum samples are required, one at the first sign of infection and one a week or more later; delay of the first sampling can prevent detection of seroconversion. IgM is induced very soon after IBV infection and transiently, and thus detection of IBV-specific IgM is indicative of recent infection (reviewed by 51, 54). Some results are conflicting; detection of IgM is not routinely done as yet.

VN and HI tests for IBV antibodies generally are considered to be type-specific, although there are cross-reactions between serotypes, especially in the HI test. Sera collected after single infections, including vaccination, can be strain-specific, let alone serotype-specific (51, 65, 99). This may limit the use of the HI test to monitor vaccine response. For example, an HI test with the M41 as antigen performed poorly when used to detect antibodies following vaccination with H120, although both viruses are of the same serotype, as defined by VN tests.

Cross-reactions are most evident when sera are collected after a field or experimental infection of broilers that have previously been vaccinated against IB—the usual situation in the field—and in sera from layers, which may have had multiple IB infections in addition to several IB vaccinations. Notwithstanding the low cost, simple equipment and speed of the HI test makes it a very useful procedure for routine diagnosis; the limitations must simply be borne in mind, and alternative analytical techniques should be available when doubt arises (50).

A monoclonal antibody-based blocking or competition ELISA has been described for the detection of antibodies to North American serotypes Massachusetts and Arkansas in the sera of experimentally inoculated chickens (99). Chicken serum containing IBV antibodies was added to virus-coated microtiter plates

followed by serotype-specific monoclonal antibody. Chicken antibody specific to an IBV serotype blocked binding of monoclonal antibody specific to the same serotype, and the blocking was proportional to the concentration of antibody in chicken serum. Specificity of the blocking ELISA compared well with that of the VN test.

Differential Diagnosis

Infectious bronchitis may resemble other acute respiratory diseases such as Newcastle disease (ND), infectious laryngotracheitis, low pathogenicity avian influenza, and infectious coryza. Newcastle disease caused by velogenic viscerotropic or neurotropic strains of paramyxovirus type 1 produces much higher mortality than IB. Lentogenic ND infections with pneumotropic strains and low pathogenicity strains of avian influenza produce mild to moderate respiratory disease with low mortality and, thus, may resemble IB. Laryngotracheitis tends to spread more slowly in a flock, but respiratory signs may be more severe than with IB. Infectious coryza can be differentiated on the basis of facial swelling that occurs only rarely in IB. Production declines and shell quality problems in flocks infected with the egg drop syndrome (EDS) adenovirus are similar to those seen with IB, except that internal egg quality is not affected in the case of EDS (57).

Intervention Strategies

Management Procedures

Ideal management includes strict isolation and repopulation with only day-old chicks, following the cleaning and disinfection of the poultry house. Current commercial production methods, which include limited clean-out and disinfection between broiler flocks, and multiple flock ages on a layer farm complex, make control more difficult and have necessitated the use of immunization to attempt to prevent production losses due to IB. Immunization is also used in isolated single-age laying flocks to prevent the heavy production losses that may result from an IBV infection of a susceptible flock during the laying cycle.

Vaccination

Types of Vaccine

Both live and inactivated virus vaccines are used in IB immunization. Live vaccines are used in meat type (broiler) chickens and for the initial vaccination and priming of breeders and layers pullets. Infectious bronchitis virus strains used for live vaccines are attenuated by serial passage in embryonating chicken eggs (see "Pathogenicity") (84, 104, 123). Extensive passage is avoided to prevent a reduction in immunogenicity. The degree and stability of such attenuation probably varies among vaccines. Evidence that some vaccines increased in virulence after backpassage in chickens (83) demonstrates the potential for enhancement of virulence of such vaccines by a cyclic infection in a flock. The use of fractional doses of IB attenuated vaccines has been associated with enhancing cyclic infections in a flock and an increase in vaccine-associated virulence.

Massachusetts serotype vaccines are very commonly used in many countries. If virus of this type is isolated from a Massachusetts-vaccinated flock with respiratory clinical signs, there may be a tendency to believe that it is simply re-isolated vaccinal virus and that some other serotype must be responsible for the disease. This may not be the case; virulent virus of the Massachusetts serotype is still present in many countries. Vaccine strains are selected to represent the antigenic spectrum of isolates in a particular country or region. The Massachusetts (M41) strain, H120, and other vaccines of the Massachusetts serotype are used widely around the world. New types subsequently may be included when the prevalence of the new type is established. In the United States, strains belonging to Massachusetts, Connecticut, and Arkansas (Ark) serotype are widely used; whereas other serotypes such as DE072 are used regionally. In some European and Asian countries, strains of serotypes D274, D1466, and 4/91 (also known as 793/B and CR88), first isolated in Europe, are used in addition to H120 and other Massachusetts vaccines. Some countries in Asia also have vaccines based on local strains (117). Only vaccines based on local isolates are permitted in Australia (104, 169).

Despite the application of Ark DPI vaccine in the Delmarva region of the United States, there have been infections by Ark serotype strains that have caused economic loss. Variants of the Ark serotype may have arisen by selection, in chickens in the field, of minor virulent populations within the attenuated vaccinal virus preparation (137). The authors suggested that the Ark DPI should be used all year round, rather than seasonally as is sometimes the case with this vaccine, as virulent subpopulations may then be less likely to rise to prominence.

IBV vaccine has been shown to interfere with the replication of attenuated avian pneumovirus, although it did not prevent the induction of protective immune responses by the pneumovirus (40). Vigorous growth of both vaccinal and field strains of IBV probably reduce the replication and, hence, efficiency of detection, of pneumovirus in the field (26).

Inactivated oil-emulsion vaccines (12) are administered in breeders and layers prior to the onset of egg production. Layer pullets may be vaccinated between 10 and 18 weeks depending on the immunization program. The seed viruses for inactivated vaccines need not be attenuated as they will be inactivated using formalin, beta propiolactone, or other suitable inactivant. Mineral oil adjuvants are commonly used to formulate the vaccine (91). The efficacy of inactivated vaccines depends heavily on proper priming with a live vaccine(s). Inactivated vaccines must be administered to birds individually, by intramuscular or subcutaneous injection. Inactivated vaccines induce serum antibody production and provide protection to internal tissues, kidney and reproductive tract. In contrast to live vaccines, inactivated vaccines are not nearly as effective at preventing infection of the respiratory tract following challenge with the homologous virulent virus. Inactivated vaccines do reduce the incidence of virus present in the respiratory tract of challenged chickens and, thus, limit transmission to other susceptible birds (110).

New "variant" strains may be used to prepare inactivated autogenous vaccines for controlling IB without the risks of using a live variant that could spread to and potentially cause disease in

nearby flocks. Inactivated variant vaccines may offer better protection against challenge with the virulent live variant IBV than inactivated vaccines containing standard serotypes such as Massachusetts and Connecticut (110).

Application Methods

Live vaccine combinations of IBV with NDV are used frequently. If the IBV component is in excess, there may be an interference with the NDV response (162). No similar interference with the IBV response has been reported.

Experimental administration of live vaccine can be individually by eye drop, intratracheal (7), or intranasal. An embryonal injection method has also been used experimentally. Commercial mass application methods include coarse spray (7), aerosol, and drinking water (146). Mass administration methods are popular because of convenience, but problems in attaining uniform vaccine application can occur, and the aerosol method may cause more severe respiratory reactions. Close attention must continually be paid to the settings and maintenance of the spraying equipment. Vaccines applied by the drinking water method are susceptible to inactivation by sanitizers added to control bacterial and fungal contamination of the watering system. Removal of those sanitizers prior to vaccination and the incorporation of powdered skim milk at a 1:400 concentration have been shown to stabilize the virus titer during vaccine administration (70).

Inactivated vaccines require injection of individual birds. In the poultry industry, these vaccines are administered between 10 and 18 weeks of age and are given 2–4 weeks after a series of 3–4 priming immunizations with live vaccine. They are often given in combination with other inactivated vaccines.

Broilers are most commonly vaccinated with live IB vaccine in the hatchery (i.e., at one day of age). A second vaccine of the same or different serotype may be given at 10–18 days of age. Experiments examining the efficacy of day-old IB vaccination are described in the section "Passive Immunity." Broiler breeders and commercial layers are likely to be first inoculated with live IB vaccine at about 2–3 weeks of age. Timing of initial immunization varies due to titer of maternal antibody in chicks and vaccination methods used. Schedules of subsequent immunization at 7–12 or 16–18 weeks of age, and at point of lay, vary with flock management and needs for control of IB, as well as other flock diseases. In the United States, many commercial egg-type chickens are vaccinated at 8–10-week intervals throughout the laying cycle with Massachusetts vaccine administered by drinking water or aerosol.

As yet no IB vaccines have been applied *in ovo*; all reduce hatchability to uneconomic levels.

Genetic manipulation of the IBV genome (17, 78) offers the prospect that a virulent IBV could be attenuated very precisely, as well as enabling spike gene swapping to make a vaccine suitably for a new serotype (16).

An experimental recombinant vaccine using the S1 gene from the Vic S strain of IBV was constructed in fowl adenovirus (FAV) serotype 8. Broiler chickens orally vaccinated at hatching or six days later were protected when given a homologous (Vic) or heterologous (N1/62) strain challenge at day 35 of age. The construction of a recombinant FAV expressing S1 demonstrates the potential of an alternative vaccination strategy against IBV (94).

Future Vaccines

There are probably dozens, at least, of sero/genotypes of IBV awaiting discovery, which will pose challenges to the poultry industry and to vaccine developers. It will only be economically feasible to develop new vaccines against a tiny number of new types of IBV. Therefore, control of IB will continue to involve "juggling" with a very small selection of vaccines, plus good management. Given that IBV replicates at a great many epithelial surfaces, it is possible that some of the yet-to-be-discovered IBV types will be associated with new clinical manifestations. Given what we have learned recently about IBV and IBV-like viruses in other species, we can speculate that some new IBV types may originate in other bird species, possibly requiring adaptation to chicken to become pathogenic. Genetic manipulation systems for IBV offer the prospect of a new era of genetically more defined and stable vaccines, and possibly ones for application *in ovo*.

Treatment

No specific treatment exists for IB. Provision of additional heat to eliminate cold stress, elimination of overcrowding, and attempts to maintain feed consumption to prevent weight loss are flock management factors that may help reduce losses from IB. Treatment with appropriate antibacterials may be indicated to aid in reducing the losses from airsacculitis resulting from infection by secondary bacterial pathogens. Electrolyte replacers, supplied in the drinking water, are recommended and were used in Australia to compensate for the acute loss of sodium and potassium and to thereby reduce losses from nephritis. The recommended concentration for treatment is 72 mEq of sodium and/or potassium, with at least one-third in the citrate or bicarbonate salt form (46).

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Chapter 5

Laryngotracheitis

J. S. Guy and M. Garcia

Introduction

Laryngotracheitis (LT) is a viral respiratory tract infection of chickens that may result in severe production losses due to mortality and/or decreased egg production. Severe epizootic forms of infection are characterized by signs of respiratory depression, gasping, expectoration of bloody mucus, and high mortality. Mild enzootic forms of infection are encountered increasingly in developed poultry industries and manifest variously as mucoid tracheitis, sinusitis, conjunctivitis, general unthriftiness, and low mortality. Laryngotracheitis virus (LTV) is a pathogen normally selected for exclusion from specific-pathogen-free chicken flocks.

Economic Significance

The economic significance of LT has not been precisely determined. However, the poultry industry of the United States can expect to experience multimillion dollar losses each year as a consequence of LTV-induced mortality and decreased egg production, and similar losses likely occur in intensive poultry industries of other countries.

Public Health Significance

No evidence suggests that LTV is transmissible to human beings and other mammals.

History

The disease was first described in 1925 (126), but some reports indicate that it may have existed earlier (14, 78). It has been given several different names including laryngotracheitis, infectious laryngotracheitis, and avian diphtheria. Some early investigators also referred to the disease as infectious bronchitis. The term laryngotracheitis was used as early as 1930 (15, 66) and the name infectious laryngotracheitis was adopted in 1931 by the Special Committee on Poultry Diseases of the American Veterinary Medical Association. The cause of LT was first shown to be a filterable virus by Beaudette (18). Subsequently, in 1934, Brandly and Bushnell devised a method for immunization of chickens based on application of virulent virus to the cloaca (26). Laryngotracheitis was the first major avian viral disease for which an effective vaccine was developed.

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Etiology

Classification

Laryngotracheitis virus is classified as a member of the genus *Iltovirus* within the family *Herpesviridae*, subfamily *Alphaherpesvirinae* (44). The virus has been shown to be genetically distinct from other alphaherpesviruses based on DNA sequence analyses, and these findings have led to its recent classification as a single member of the genus *Iltovirus* (44, 127). The virus is taxonomically identified as *Gallid herpesvirus 1* (44, 155).

Morphology

Electron micrographs of LTV-infected chicken embryo cell cultures demonstrate the presence of icosahedral viral particles similar in morphology to herpes simplex virus (Fig. 5.1). Watrach *et al*. (184) described the hexagonal nucleocapsids of LTV to be 80–100 nm in diameter. The nucleocapsids have icosahedral symmetry and are composed of 162 elongated hollow capsomeres (43, 184).

The complete virus particle has a diameter of 195–250 nm and consists of an irregular envelope surrounding the nucleocapsid. Nucleocapsids may be observed within enveloped particles when these are penetrated by stain; if stain does not penetrate particles, they may be difficult to distinguish from cell debris. The envelope contains viral glycoprotein spikes as fine projections on its surface.

Chemical Composition

The nucleic acid of LTV is composed of DNA with a buoyant density of 1.704 g/mL, a value consistent with other herpesviruses (141). The molecular weight of LTV DNA is approximately 100×10^6, with the genome having two isomeric forms (119, 121). Laryngotracheitis virus DNA has been reported to have a guanine plus cytosine ratio of 45% (141), a value lower than many other animal herpesviruses. The DNA genome consists of a linear 155-kb double-stranded molecule composed of unique long (UL) and unique short (US) regions flanked by inverted repeats (97, 122). Recently, the complete nucleotide sequence of the LTV genome was assembled from 14 different published sequences (173). The assembled LTV genome was shown to consist of a 148-kb molecule having a UL region of 113 kb, and a US region of 13 kb; the UL and US regions were shown to be flanked by two 11-kb inverted repeats. The LTV genome contains a total of 77 predicted open reading frames; 62 of these are located in the UL region, 9 in the US region, and 3 in the inverted repeats.

Early studies using partial genome sequence data from the LTV thymidine kinase gene and upstream overlapping genes demonstrated DNA homology between LTV and various other alphaherpesviruses (67, 106). Subsequently, using sequence data for the entire LTV genome, and other herpesvirus genomes, revealed that Psittacid herpesvirus 1 (PsHV-1) and LTV represent a unique clade of avian alphaherpesviruses that are distinct from Marek's disease-like viruses (*Mardivirus*) (173). The LTV and the PsHV-1 genomes share a unique block of five open reading frames, and a large internal inversion in the unique long region similar to an inversion previously found in the genome of the pseudorabies virus, a porcine alphaherpesvirus (182, 198).

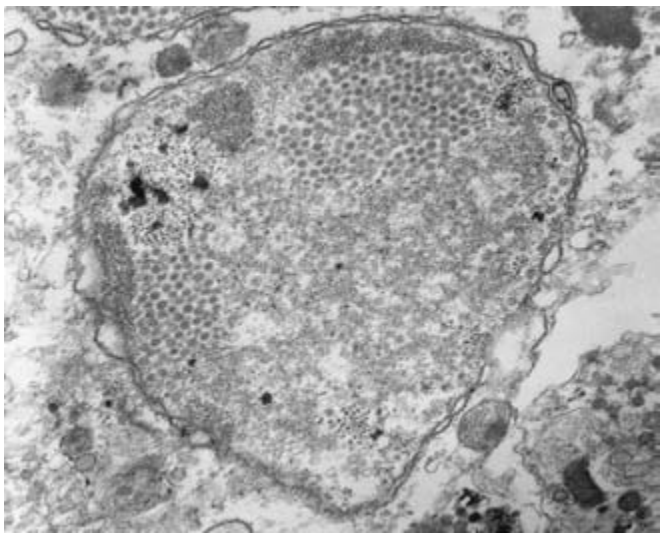
The glycoproteins of LTV, like other herpesviruses, are responsible for stimulating humoral and cell-mediated immune responses (192). Early studies by York *et al*. (195, 197) identified five major envelope glycoproteins with molecular weights of 205, 160, 115, 90, and 60 kD to be the major immunogens of LTV. Subsequently, characterization of LTV glycoproteins utilizing monospecific antisera or monoclonal antibodies has been undertaken in several laboratories. Several glycoproteins that are homologous to those of human herpes simplex virus have been identified in LTV; these are designated glycoprotein B (gB) (142), gC (114, 180), gN (56), gM (55, 56), gG (117), and gJ (180). The gJ protein was initially identified as a 60 kDa protein and named gp60 (118). Later work indicated that gJ was expressed as multiple proteins of 85, 115, 160, and 200 kD sizes, and gC was expressed as a single 60 kD protein.

Recent studies with viruses having deletions in genes coding for gJ, gM, and gN genes (LTV deletion mutant viruses) have shown that these glycoproteins are not essential for virus replication (49, 56, 58). Another study examining a LTV with a double gI/gE gene deletion demonstrated that these two viral glycoproteins are essential for virus replication (48).

Virus Replication

Replication of LTV appears to be similar to that of other alphaherpesviruses such as pseudorabies virus and herpes simplex virus (68, 143, 156). The virus initiates infection by attachment to cell receptors followed by fusion of the envelope with the host cell plasma membrane. The nucleocapsid is released into the cytoplasm and transported to the nuclear membrane; viral DNA is released from the nucleocapsid and migrates into the nucleus through nuclear pores. Transcription and replication of viral DNA occur within the nucleus.

Transcription of LTV DNA occurs in a highly regulated, sequentially ordered cascade similar to that of other alphaherpesviruses (86, 143). Approximately 70 virus-coded proteins are produced; several are enzymes and DNA-binding proteins that regulate viral DNA replication, but most are viral structural proteins. Viral DNA replication occurs by a rolling circle mechanism with the formation of concatemers (19). DNA concatemers are cleaved into monomeric units and packaged into preformed nucleocapsids within the nucleus. DNA-filled nucleocapsids acquire an envelope by migration through the inner lamellae of the nuclear membrane (68). Enveloped particles then migrate through the endoplasmic reticulum and accumulate within vacuoles in the cytoplasm (68). Enveloped virions are released by cell lysis or by vacuolar membrane fusion and exocytosis.



5.1. Electron micrograph of laryngotracheitis virus-infected cell. Aggregates of virus particles form an inclusion body in nucleus of infected chicken embryo kidney cell. Note the peripheral accumulations of chromatin and centrally located amorphous material; the latter forms part of the inclusion body. ×18,500. (Watrach)

Susceptibility to Chemical and Physical Agents

Laryngotracheitis virus is sensitive to the effects of lipolytic agents such as chloroform and ether (54, 130). Laryngotracheitis virus infectivity survives for several months when stored at 4°C in suitable diluents such as glycerol or nutrient broth. However, the thermostability of LTV infectivity has been the subject of reports which vary considerably. For example, the infectivity of LTV has been reported to be rapidly inactivated by heat when exposed to 55 °C for 15 minutes or 38 °C for 48 hrs (101). Conversely, Meulemans and Halen (130) found that 1% of the infectivity of a Belgian strain was retained after 1 hr at 56 °C. Cover and Benton reported that LTV was destroyed in 44 hr at 37°C in tracheal tissues within chicken carcasses or in chorioallantoic membranes (CAMs) after 5 hr at 25 °C (39). These results are, however, greatly at variance with several earlier reports (101) that indicated the capability of LTV infectivity to survive in tracheal exudates and chicken carcasses for periods of 10–100 days at ambient temperatures of 13–23 °C. Additional studies are needed to resolve these discrepancies.

A solution of 3% cresol or 1% lye will inactivate LTV in less than 1 minute; laboratory bench surfaces can be readily decontaminated with commercial iodophors or halogen-detergent mixtures. Studies with microaerosolized hydrogen peroxide have indicated that complete inactivation of LTV infectivity was achieved with a 5% hydrogen peroxide mist as a fumigant for poultry house equipment (133).

Strain Classification

Antigenicity

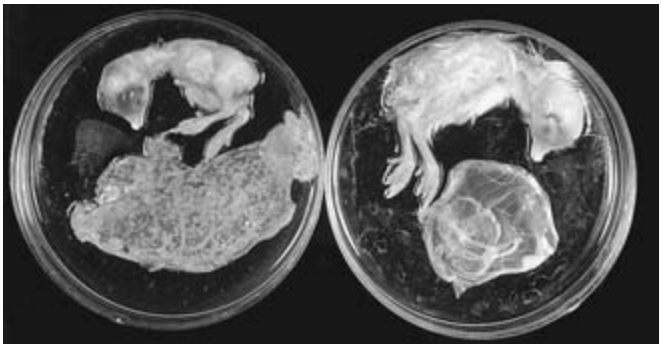
Laryngotracheitis virus strains appear to be antigenically homogenous based on virus-neutralization, immunofluorescence tests, and cross-protection studies (39, 169). However, minor antigenic variation among strains has been suggested by findings that some strains are neutralized poorly by heterologous antisera (145, 158, 169).

Molecular Classification

Molecular methods for differentiating LTV strains include restriction endonuclease analyses of viral DNA (70, 74, 119, 121), DNA hybridization assays (120), polymerase chain reaction (PCR) procedures combined with restriction fragment length polymorphism (RFLP) analyses of amplified DNA (PCR-RFLP) (31, 38, 42, 60, 65, 76, 115), PCR-RFLP combined with gene sequencing (75), and gene sequencing alone (136). Restriction endonuclease cleavage of viral DNA and electrophoretic separation of DNA fragments have been shown to distinguish different LTV strains (119, 121). Restriction endonuclease analysis of LTV DNA has been used extensively in epidemiological studies of field outbreaks to differentiate modified-live vaccine viruses from non-vaccine (wild-type) LTV strains (7, 70, 74, 107, 109).

Reciprocal DNA:DNA hybridization using cloned DNA fragments has been shown to discriminate LTV strains (120). However, additional testing of this method is needed to determine the accuracy of this method for differentiating LTV strains.

Recent advances in our understanding of the LTV genome based on nucleotide sequence studies, and the assembly of the complete LTV genome based on published sequences (173), have provided the basis for strain differentiation using genetic differences identified by PCR-RFLP and/or gene sequencing. Several PCR-RFLP assays have been described that differentiate vaccine and nonvaccine LTV strains (31, 38, 42, 60, 65, 76). Polymerase chain reaction-RFLP analysis of the infected cell protein 4 (ICP4) gene was shown to discriminate between vaccine and nonvaccine isolates from Taiwan (31) and Northern Ireland (65). In both reports, outbreak-related viruses obtained prior to the introduction of modified-live LTV vaccines were identified as nonvaccine virus, while vaccine viruses were identified as the cause of outbreaks after the implementation of LTV vaccination. Using a single nucleotide polymorphic site previously identified in the ICP4 gene (65), a PCR-RFLP assay allowed the detection and differentiation of vaccine and nonvaccine viruses directly from field cases in the United Kingdom (42). In another study, PCR-RFLP combined with nucleotide sequence analysis of the glycoprotein G (gG) and the thymidine kinase (TK) genes allowed the differentiation of non-vaccine from vaccine viruses in Korea (75), and the analysis of both these genes allowed the identification of a viral isolate that might have originated from a recombination event between a vaccine and a nonvaccine virus. Kirkpatrick *et al.* (115) utilized PCR-RFLP to differentiate among isolates of LTV strains in Australia. They showed that reliable differentiation of LTV strains required the examination of multiple genes (gG, TK, ICP4, ICP18.5 genes, open reading frame [ORF] B-TK) and that most of the recent LT outbreaks in Australia were not caused by vaccine viruses (115).



5.2. Chicken embryos at 14 days of age. Normal embryo and chorioallantoic membrane (CAM) (*right*). Laryngotracheitis virus-infected embryo is stunted, and CAM has numerous foci of necrosis and cell proliferation (*left*).

Nucleotide sequence analyses of the UL47 and gG genes allowed the identification of vaccine and nonvaccine viruses involved in LT outbreaks in Ontario (136).

Pathogenicity

Naturally occurring LTV strains vary in virulence from highly virulent strains that produce high morbidity and mortality in exposed chickens to strains of low virulence that produce mild-to-inapparent infection (39, 101, 144, 145, 167, 174). Laryngotracheitis virus strains also were shown to differ based on virulence for chicken embryos (94), plaque size and morphology in cell culture (157), and plaque size and morphology on chorioallantoic membrane (CAM) of embryonated chicken eggs (145). Differentiation of LTV strains of varying virulence, particularly wild-type and modified-live vaccine viruses, is an important practical problem. Assessment of mortality patterns in embryonated chicken eggs was proposed as a biological system for differentiating LTV strains (94) as they found that mortality patterns correlated closely with virulence.

Laboratory Host Systems

Laryngotracheitis virus might be propagated in embryonated chicken eggs and a variety of avian cell cultures. In embryonated chicken eggs the virus causes formation of opaque plaques on the CAM resulting from necrosis and proliferative tissue reactions (Fig. 5.2). Chorioallantoic membrane plaques generally have opaque edges and a central depressed area of necrosis. Plaques can be observed as early as 2 days postinoculation (PI) and embryo deaths occur 2–12 days PI. Survival time of inoculated embryos decreases with additional egg passages (23, 25, 28).

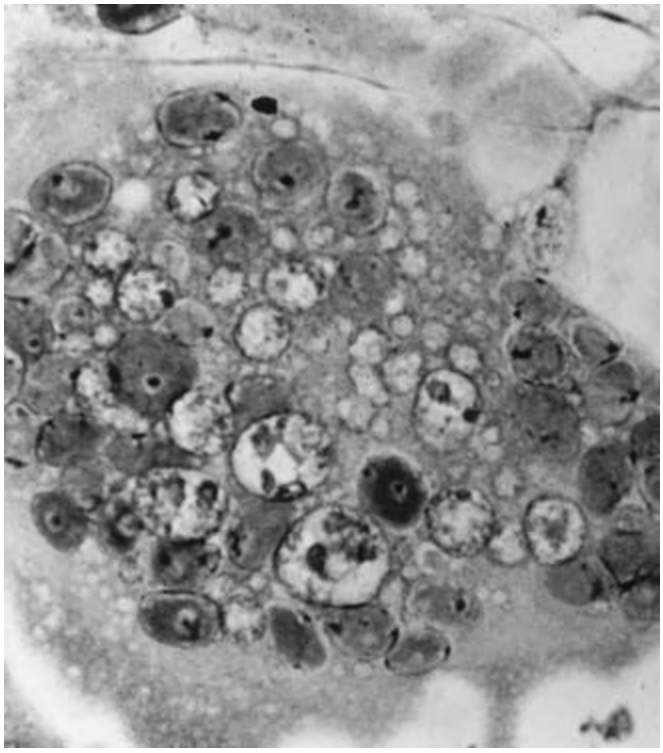
Laryngotracheitis virus has been propagated in a variety of avian cell cultures including chicken embryo liver (CEL), chicken embryo lung, chicken embryo kidney (CEK), and chicken kidney (CK) cell cultures (33, 88, 128, 129). Hughes and Jones (88) compared several different laboratory host systems for efficiency of LTV isolation and propagation. The CEL and CK cells were found to be the preferred culture systems, with CEK cells, chicken embryo lung cells, and CAM inoculation of em-

bryonated chicken eggs being less sensitive. Chicken embryo fibroblast cells, Vero cells, and quail-origin cells have been determined to be poor substrates for LTV propagation (88, 162).

Viral cytopathology may be observed in cell culture as early as 4–6 hr PI with a high multiplicity of infection. Cytopathology consists of increased refractiveness and swelling of cells, chromatin displacement, and rounding of the nucleoli. Cytoplasmic fusion results in formation of multinucleated giant cells (syncytia) (Fig. 5.3). Intranuclear inclusion bodies can be detected as early as 12 hr PI, with the highest concentration occurring 30–36 hr PI. Large cytoplasmic vesicles develop in the multinucleated cells, which become more basophilic as cells degenerate (151).

Laryngotracheitis virus also may be propagated in avian leukocyte cultures. Initially, LTV was shown to replicate in avian leukocyte cultures derived from chicken buffy coat (34), and later the virus was shown to replicate in macrophage cultures obtained from bone marrow and spleen (27). Calnek *et al*. (30) determined that macrophage cultures were as susceptible to LTV infection as CK cells, but replication of most LTV strains examined was restricted. Both cell genotype and virus genotype influenced the extent of restriction of virus replication. Other cell types including lymphocytes, thymocytes, buffy-coat leukocytes, and activated T cells were either refractory or nearly refractory to LTV infection.

Laryngotracheitis virus also has been shown to replicate in LMH cells, a continuous avian cell line derived from a chemically induced chicken liver tumor (162). However, propagation of LTV in LMH cells requires adaptation, thus this cell line is unsuitable for diagnostic purposes involving primary isolation. They may, however, be useful for other purposes; for example, in research laboratories studying virus-host cell interactions.



5.3. Chicken embryo kidney cell monolayer, 72 hr after inoculation with laryngotracheitis virus. A multinucleated giant cell (syncytium) has formed with many nuclei containing inclusion bodies. May-Grünwald-Giemsa, ×320.

Pathobiology and Epidemiology

Incidence and Distribution

Laryngotracheitis virus has been identified in most countries and remains a serious disease wherever susceptible poultry populations occur, especially in large numbers (22). In areas of intensive production and large concentrations of poultry such as in the United States, Europe, China, Southeast Asia, and Australia, LT is usually well controlled in layers by the use of modified-live virus vaccines. For intensive broiler production, the short growth cycle and high level of quarantine on sites can reduce the need for prophylactic vaccination. Within developed countries, LT viruses have tended to persist as endemic infections within backyard and fancier chicken flocks.

Natural and Experimental Hosts

The chicken is the primary natural host of LTV. Although the disease affects all ages, the most characteristic signs are observed in adult birds. Viral multiplication is limited to respiratory tissues, with little or no evidence of viremia (11, 85).

Several workers have described a form of LT in pheasants and pheasant-chicken crosses (41, 87, 111). Winterfield and So (189) were able to induce lesions in the upper respiratory tract of young turkeys. They also reported the isolation of LTV from the trachea of a peafowl. Previous failures to infect turkeys (24, 166) would indicate an age-dependent resistance in this species. Starlings, sparrows, crows, doves, ducks, pigeons, and guinea fowl appear to be refractory to LTV (16, 26, 166); however, Yamada *et al*. reported subclinical infection and seroconversion in ducks (190).

Embryonated eggs of turkeys and chickens are susceptible to LTV, while duck eggs are susceptible to a lesser degree (101, 190) and eggs of guinea fowl and pigeons are not susceptible.

Transmission, Carriers, Vectors

Natural portals of entry for LTV are through the upper respiratory and ocular routes (17, 18). Ingestion can also be a mode of infection, although exposure of nasal epithelium following ingestion is required with this route (154). Transmission occurs more readily from acutely infected birds than through contact with clinically recovered carrier birds (see "Pathogenesis").

Mechanical transmission can occur by use of contaminated equipment and litter (18, 50, 64, 113). Egg transmission of virus contained in the interior or exterior of the egg has not been demonstrated.

Incubation Period

Clinical signs generally appear 6–12 days following natural exposure (110, 165). Experimental inoculation via the intratracheal route results in a shorter incubation period of 2–4 days (20, 99, 165).



5.4. Dyspnea exhibited by an adult chicken with laryngotracheitis. Note dried blood exudate around nostril and along the lower beak (*arrow*). (Munger)

Clinical Signs

Laryngotracheitis virus causes an acute respiratory disease in chickens. Characteristic clinical signs include nasal discharge and moist rales followed by coughing and gasping (Fig. 5.4) (14, 110). Marked dyspnea and expectoration of blood-stained mucus is characteristic of severe epizootic forms of the disease (14, 78, 79, 98, 165).

Severe epizootic forms of LT were described commonly in earlier years. However, in recent years mild enzootic forms of LT have been more commonly observed in the intensive poultry producing areas of Europe, Australia, New Zealand and the United States (39, 123, 145, 165, 167, 185). Clinical signs associated with mild enzootic forms include unthriftiness, decreased egg production, watery eyes, conjunctivitis, swelling of infraorbital sinuses, mild tracheitis, persistent nasal discharge, and hemorrhagic conjunctivitis.

The course of the infection varies with the severity of lesions. Generally, most chickens recover in 10–14 days, but extremes of 1–4 weeks have been reported (14, 78).

Morbidity, and Mortality

Severe epizootic forms of the disease cause high morbidity (90–100%) and variable mortality; mortality can vary from 5% to 70% but usually is in the range of 10–20% (14, 78, 165). Mild enzootic forms of the disease described in Great Britain, Australia, the United States, and New Zealand result in morbidity as low as 5% and very low mortality (0.1–2%)(39, 123, 145, 150, 167, 185).

Pathology

Gross

Gross lesions may be found in the conjunctiva and throughout the respiratory tract of LTV-infected chickens, but they are most consistently observed in the larynx and trachea. Tissue changes in tracheal and laryngeal tissues may be mild, consisting only of excess mucus (123), or severe with hemorrhage and/or diphtheritic changes. In severe forms, mucoid inflammation is observed early in infection with degeneration, necrosis, and hemorrhage occurring in later stages. Diphtheritic changes are common and may be seen as mucoid casts that extend the entire length of the trachea. In other cases, severe hemorrhage into the tracheal lumen may result in blood casts (Fig. 5.5A), or blood may be mixed with mucus and necrotic tissue. Inflammation may extend down the bronchi into the lungs and air sacs.

In mild forms of LT, gross lesions may consist only of edema and congestion of conjunctiva and infraorbital sinuses, and mucoid tracheitis (45, 123).

Microscopic

Microscopic changes vary with the stage of the disease (see Figs. 5.5B–F). Early microscopic changes in tracheal mucosa include the loss of goblet cells and infiltration of mucosa with inflammatory cells. As the viral infection progresses, respiratory epithelial cells enlarge, lose cilia, and become edematous. Multinucleated cells (syncytia) are formed and lymphocytes, histiocytes, and plasma cells migrate into the mucosa and submucosa after 2–3 days. Later, cell destruction and desquamation result in a mucosal surface either covered by a thin layer of basal cells or lacking any epithelial covering; blood vessels within the lamina propria may protrude into the tracheal lumen. Hemorrhage may occur in cases of severe epithelial destruction and desquamation with exposure and rupture of blood capillaries.

Intranuclear inclusion bodies are found in epithelial cells by 3 days PI (146). Inclusion bodies generally are present only in the early stages of infection (1–5 days) (73, 178); they disappear as infection progresses, a result of necrosis and desquamation of epithelial cells.

Ultrastructural

Electron microscopic studies have shown that the first cellular changes occur in the nucleus of epithelial cells during formation of viral capsids (151). Viral capsids bud through the nuclear membrane, acquiring lipid envelopes, and aggregate into large masses within vacuoles in the cytoplasm. The cloudy swelling observed in light microscopic studies of early cellular changes has been associated with the presence of these large masses of viral particles in the cytoplasm (183).

Pathogenesis of the Infectious Process

Laryngotracheitis virus infection of susceptible chickens results in virus replication in epithelium of larynx and trachea, and potentially other mucous membranes such as conjunctiva, respiratory sinuses, air sacs and lungs. Laryngotracheitis virus strains generally are highly cytolytic in these tissues, particularly trachea, potentially resulting in severe epithelial damage and hemorrhage.

Several studies have independently confirmed that infectious virus usually is present in tracheal tissues and tracheal secretions for 6–8 days PI (11, 85, 147, 154); the virus may remain at very low levels up to 10 days PI (187). No clear evidence exists for a viremic phase of infection. Extratracheal spread of LTV to trigeminal ganglia was first reported by Bagust *et al*. (11); the

virus was detected in trigeminal ganglia of chickens 4–7 days after tracheal exposure with a virulent Australian LTV strain. Reactivation of latent LTV from the trigeminal ganglia 15 months after vaccination of a flock has since been reported from Germany (104). Williams *et al*. (187), with the use of polymerase chain reaction (PCR) technology, have confirmed that the trigeminal ganglion is the principal site of LTV latency. Hughes *et al*. (90) reported the re-excretion of LTV from latently infected chickens following the stress of re-housing and the onset of reproduction.

Clinically inapparent LTV infection of the respiratory tract is a major feature of LTV persistence. Pioneering observations by Komarov and Beaudette (116) and Gibbs (63), who collected laryngeal and tracheal swabs and inoculated susceptible chickens, indicated a "field" carrier rate of approximately 2% for periods up to 16 months after a disease outbreak. In studies with tracheal organ cultures explanted from chickens experimentally infected with Australian wild-type LTV and vaccine strains, latent tracheal infections were demonstrated for similar periods in 50% or more of infected chickens (8, 177). In a recent study using PCR it was determined that latent infections of the trachea and the trigeminal ganglion can be simultaneously established by vaccine and challenge strains early after experimental infection (76). Repeated tracheal swabbing of small groups of chickens that had been experimentally infected with either a mildly pathogenic United Kingdom field strain or LT vaccine strains detected intermittent and apparently spontaneous shedding of LTV between 7 and 20 wk after infection (89, 91). Treatment with immunosuppressive drugs (e.g., cyclophosphamide, dexamethasone) has not been successful in reactivating latent LTV (8, 89, 90).

Immunity

Active

A variety of immune responses are generated following LTV infection (102). Virus-neutralizing antibodies become detectable within 5–7 days PI, peak around 21 days PI, then wane over the next several months to low levels. Virus-neutralizing antibodies may be detectable for a year or more (84). Antibodies may be detected in tracheal secretions from approximately 7 days PI (8, 196) and plateau at days 10–28 PI. The numbers of IgA- and IgG-synthesizing cells in the trachea increased substantially in experimentally infected chickens between days 3 and 7 PI (196). Cell-mediated immunity (CMI) has not been extensively studied owing to the complexity of CMI studies; however, delayed-type hypersensitivity responses to LTV have been demonstrated (197). The duration of CMI responses to LTV following infection is not known.

Humoral immune responses to LTV, although associated with infection, are not the primary mechanism of protection, and a poor correlation generally has been found between serum antibody titers and immune status of flocks (102). In addition, Fahey and York (51), with the use of bursectomized chickens, have demonstrated that mucosal antibody is not essential in preventing replication of virus in vaccinated chickens. The principal mediator of LT resistance is the local cell-mediated immune response in the trachea (51). Bursectomized and cyclophosphamide-treated chickens fail to mount humoral immune responses following LT vaccination but develop full immunity (51, 152). Fahey *et al*. (53) demonstrated that LT resistance could be transferred using spleen cells and peripheral blood leukocytes from congenic immune donors.

Sinkovic (171) and Fahey *et al*. (52) determined that susceptibility of chickens to LTV declined with age. Sinkovic (171) also found that meat-type males were more susceptible than meat-type females and that high environmental temperature (35°C) resulted in higher mortality from LTV infection in heavy adult breeds than in light adult breeds.

Passive

Maternal antibody to LTV is transmitted to offspring via the egg (21). However, maternal antibody does not confer protection to infection or interfere with vaccination (52, 171).

Diagnosis

In general, LT diagnosis requires laboratory assistance as other respiratory pathogens of poultry can cause similar clinical signs and lesions. Only in cases of severe acute disease with high mortality and expectoration of blood can LT be reliably diagnosed on the basis of clinical signs. Otherwise, diagnosis of LT should be based on one or more confirmatory laboratory diagnostic procedures including detection of intranuclear inclusion bodies, virus isolation, detection of LT virus antigens in tracheal tissues or respiratory mucus, detection of LT virus-specific DNA, or serology (176).

Histopathology

Laryngotracheitis is characterized by the development of pathognomonic intranuclear inclusion bodies in respiratory and conjunctival epithelial cells. Intranuclear inclusion bodies may be detected in tissues stained with Giemsa or hematoxylin and eosin. Cover and Benton (39) reported that the choice of fixative was important, and that a fixative having a low pH was required for detection of inclusion bodies. Diagnosis of LT based on demonstration of inclusion bodies in tissues has been shown to be considerably less sensitive than virus isolation. Keller and Hebel (108) showed that inclusion bodies could be detected in 57% of 60 specimens, while virus was isolated from 72% of the same specimens. Similarly, Guy *et al*. (73) found that histopathologic detection of inclusion bodies was a highly specific method for diagnosis of LT when compared with virus isolation, but sensitivity was poor.

Rapid methods for histopathologic identification of LTV inclusion bodies have been described by Pirozok *et al*. (140) and Sevoian (168); both techniques require as little as 3 hr for preparation of tissues as compared with 24–48 hr with the use of conventional histologic processing methods. Pirozok *et al*. (140) developed a method employing Carbowax, a water-soluble embedding medium that eliminates the need for dehydration steps, thus markedly decreasing processing time. Sevoian (168) developed a procedure in which fixation and dehydration of tissues could be performed simultaneously.

Isolation and Identification of Causative Agent

Isolation of LTV may be accomplished by inoculation of suspensions of respiratory exudate, conjunctival exudate, or homogenates of appropriate tissues onto the CAM of 9–12-day-old embryonated chicken eggs or onto susceptible cell cultures (see Laboratory Host Systems). Clinical samples may include trachea, larynx, lung, conjunctiva, or exudate collected by swabbing these sites. Samples must be collected early in the course of infection, as experimental studies indicate that LTV is not detected or detected inconsistently after approximately 6 days PI (11, 73, 191). Samples should be transported promptly to the laboratory, preferably on wet ice.

The CAM route of inoculation is utilized most commonly for LTV isolation as it is the most sensitive of the embryonated egg inoculation routes and results in titers 10^2 or greater than other routes (82, 100). Chorioallantoic membrane plaques can be observed as early as 2 days PI; these generally have opaque edges and a central depressed area of necrosis (Fig. 5.2).

Chicken embryo liver cells and CK cells are the cell cultures of choice for LTV isolation. Viral cytopathology may be observed in cell culture within 24 hr PI and consists of increased refractiveness and swelling of cells, chromatin displacement, rounding of the nucleoli, and formation of multinucleated giant cells (syncytia) (Fig. 5.3). A maximum of two serial passages in CEL and CK cell cultures are required to ensure detection of LTV in clinical samples (8, 88).

In a comparison of the CAM inoculation route and a variety of cell cultures, Hughes and Jones (88) found CEL cells to be the most sensitive laboratory host system for LTV isolation, although CK cells were a satisfactory alternative. Both CEL and CK cells were superior to CAM inoculation of embryonated eggs.

Definitive identification of LTV in infected CAM material may be accomplished using histopathology, fluorescent antibody (FA) procedures, immunoperoxidase procedures (167), or polymerase chain reaction (PCR) procedures. Electron microscopy, FA, or IP may be used to identify LTV in infected CEL and CK cells.

A variety of different procedures have been described for identification of LTV in clinical samples; these include electron microscopy, methods for detection of viral antigens (FA, IP, enzyme-linked immunosorbent assays [ELISA]), and methods for detection of viral DNA (DNA hybridization techniques, PCR techniques). Electron microscopy has been utilized to detect LTV in tracheal scrapings (88, 179). As diagnosis is dependent upon visualization and morphologic identification of herpesviruses, this approach is successful only when large numbers of virus particles are present in clinical samples. Hughes and Jones (88) found that virus particles were observed only when clinical samples contained a minimum of $10^{3.5}$ infectious virus/ 0.1 mL.

Wilks and Kogan (186) reported the detection of LTV antigens in tracheal tissue from day 2 through day 14 PI using an FA procedure, but others (11, 85) have reported considerably shorter periods (6–8 days PI) for successful detection using FA procedures. With the use of an IP procedure, Guy *et al*. (73) were able to detect LTV antigens in frozen sections of tracheal tissues from day 1 to day 9 PI (Fig. 5.6), and the IP procedure was shown to be more sensitive than FA for detection of LTV in tissues. Timurkaan *et al*. (174) were able to detect LTV antigens in formalin-fixed, paraffin-embedded tracheal and laryngeal tissues of chickens from day 3 to day 9 PI. Similarly, Sellers *et al*. (167) utilized the IP procedure to detect LTV antigens in formalin-fixed, paraffin-embedded tissues.



5.6. Immunoperoxidase staining of tracheal epithelium of chicken, 4 days after intratracheal inoculation with laryngotracheitis virus. Staining is localized to large focal areas of the tracheal mucosa (*arrow*). ×150.

Immunoperoxidase and FA detection of LTV antigens in tissues of infected chickens require a source of LTV-specific antibody. These LTV-specific antibodies have been prepared for use in FA and IP by animal immunization procedures (11, 85, 174, 186) and by use of monoclonal antibody technology (1, 180, 197). Monoclonal antibodies are advantageous in that large quantities of highly specific antibody may be produced, potentially allowing worldwide distribution and standardization of LTV diagnostic procedures.

ELISA procedures for detection of LTV antigens in tracheal exudate have been developed (135, 191). York and Fahey (191) described an antigen capture ELISA using monoclonal antibodies to LTV. This ELISA was shown to be as accurate as virus isolation, but faster, and more accurate than either FA or agar gel precipitation tests (103) for detecting LTV.

More recently, methods for detection of LTV DNA in clinical samples have been described. Keam *et al*. (105) and Key *et al*. (112) described procedures for detection of LTV DNA utilizing dot-blot hybridization assays and cloned LTV DNA fragments labeled with digoxigenin. These procedures were shown to be highly sensitive for detection of LTV in acutely infected chickens, as well as convalescent chickens when detection was no longer possible using virus isolation and ELISA. These procedures also were shown to provide rapid methods for detection of

chickens latently infected with LTV. Abbas *et al*. (2) described a dot-blot hybridization procedure using a biotinylated DNA probe generated by PCR procedures. In situ hybridization procedures were described by Nielsen *et al*. (134) for detection of LTV DNA in tissues.

Polymerase chain reaction (PCR) procedures for detection of LTV DNA have been described by a number of research groups (2, 4, 31, 38, 42, 92, 138, 164, 170, 188). These procedures include a multiplex PCR (138) that allows detection of multiple avian respiratory pathogens, including LTV, and real-time PCR (42) that markedly improves speed of diagnosis. These procedures have been shown to be more sensitive than virus isolation. Additionally, PCR procedures allow detection of LTV in samples contaminated with other microorganisms, such as adenoviruses, that may prevent LTV isolation due to overgrowth in culture (188).

Serology

A variety of techniques for demonstration of LTV-specific antibodies in serum have been described, including agar-gel immunodiffusion (AGID), virus neutralization (VN), indirect fluorescent antibody (IFA) test, and ELISA. These procedures also may be utilized for identification of LTV in infected cell cultures and CAMs.

Burnet (29) first described a VN test to detect LTV-specific antibodies in chicken serum using embryonated chicken eggs inoculated by the CAM route with subsequent enumeration of CAM lesions. The use of cell cultures has greatly simplified these procedures, and VN antibodies may be measured by assay in cell cultures seeded in tubes, petri dishes, or microwell plates (36, 153, 157).

Enzyme-linked immunosorbent assay systems have been developed for detection and quantitation of LTV-specific antibodies using LTV-coated plates (131, 135, 194). Direct comparison of the AGID, VN, IFA, and ELISA demonstrated that all were valid systems for detecting and quantifying LTV-specific antibodies (3). Although ELISA was shown to possess slightly greater sensitivity than VN, it was comparable to IFA; AGID was the least sensitive. Both ELISA and IFA have the advantages of speed and sensitivity; however, ELISA lacks the subjectivity inherent to IFA and is more suitable for testing large numbers of sera (13).

Recently, an ELISA for detection of LTV-specific antibodies was developed that utilized a recombinant *Escherichia coli* that expressed LTV glycoproteins, gE and gp60 (32). It was shown that this recombinant-based ELISA differentiated between LTV-vaccinated and unvaccinated/unexposed chickens, but sensitivity and specificity were not reported.

Differential Diagnosis

Respiratory disease associated with LT must be distinguished from other respiratory pathogens of poultry that may cause similar clinical signs and lesions. These include the diphtheritic form of avian poxvirus and infections caused by Newcastle disease virus, avian influenza virus, infectious bronchitis virus, fowl adenovirus, and *Aspergillus* spp.

Intervention Strategies

Management Procedures

Laryngotracheitis virus infections resulting from field exposure or vaccination will result in latently infected carrier birds; thus, it is extremely important to avoid mixing vaccinated or recovered birds with susceptible chickens. Special precautions should be taken to obtain a complete history when mixing breeding stock. Use of sound biosecurity measures will avoid exposing susceptible chickens via contaminated fomites.

The importance of site quarantine and hygiene in preventing the movement of potentially contaminated personnel, feed, equipment, and birds is central to successful prevention and control of LT. Measures to control dogs, cats and rodents also should be in place (113). The persistent LT disease threat posed by backyard and exhibition poultry flocks (125, 128) should be recognized and guarded against.

Cooperative control of LT outbreaks by collaboration between government and industry is most desirable. Correctly implemented (125), this approach may obviate the need for widespread use of LT vaccine. Where outbreaks have been contained, recovered flocks should be moved for processing under quarantine as soon as possible. Experience with LT outbreaks in Pennsylvania (45, 46) indicates that this interval can be as short as 2 wk after the last clinical signs of LT are observed on a site.

For control of an LT outbreak, the most effective approach is a coordinated effort to obtain a rapid diagnosis, institute a vaccination program, and prevent further virus spread (9). Vaccination in the face of an outbreak effectively limits virus spread and shortens the duration of disease. Spread of LTV between sites can be prevented by appropriate biosecurity measures.

Laryngotracheitis virus infectivity is readily inactivated outside the host chicken by disinfectants and warm temperatures, thus carryover between successive flocks in a house can be prevented by adequate cleanup. It is recommended that all potentially contaminated carcasses, feathers, feed, water and litter should be kept within the poultry house, and the house heated to 38 °C for 100 hours. Buildings and equipment should be washed and then sprayed with disinfectants such as phenolics, sodium hypochlorite, iodophors, or a quarternary ammonium compound. All disinfectants should be used only at the dilutions recommended by the manufacturer.

Vaccination

Vaccination has proven to be a satisfactory method for developing resistance in susceptible chicken populations (see "Immunity"). Since vaccination can result in latently infected carrier birds, it is recommended for use only in geographic areas where the disease is endemic. The appropriate regulatory agency should be contacted to determine approved vaccines and vaccine application procedures.

Modified-Live Virus Vaccines

Successful immunization against LT was first accomplished by application of virulent virus to the cloaca (26). Subsequently, it was demonstrated that immunity could be provided by vaccina-

tion of chickens with attenuated (modified-live) viruses via infraorbital sinuses (169), intranasal instillation (20), feather follicles (132), eye drop (172), and orally through drinking water (161). Field strains of LTV have been attenuated by sequential passage in cell cultures (62, 95, 96) and embryonated chicken eggs (160), and via feather follicle inoculation of chickens (93).

Careful attention must be given to procedures of vaccine administration to ensure adequate immunization. Care must be taken to ensure that virus dose is sufficient to provide effective immunization. Raggi and Lee (149) found that LT vaccine must contain greater than 10^2 plaque-forming units/mL to induce satisfactory immunity when administered by routes other than the oral route. A virus concentration of 10^5 embryo infective doses was necessary for satisfactory oral vaccination (80). Modified-live LT vaccines must be handled with care in order to ensure adequate concentrations of infective virus; manufacturers' instructions for storage, resuspension, dilution, and application should be closely followed.

Administration of modified-live LT vaccine in drinking water or by spray are desirable methods for rapid, mass application of these vaccines; however, several problems have been associated with these routes of inoculation. Robertson and Egerton (154) demonstrated that administration of LT vaccines by the drinking water route can result in a high proportion of chickens that fail to develop protective immunity. Successful vaccination via the drinking water is dependent upon the vaccine virus making contact with susceptible nasal epithelial cells; this occurs via aspiration of virus through external nares or choanae. The studies of Robertson and Egerton (154) showed that this occurred infrequently in chickens vaccinated by the drinking water route. Incorrect application of LT vaccines by spray may result in adverse reactions as a result of insufficient attenuation of vaccine virus, deep penetration of respiratory tract due to small droplet size of spray (148), or excessive dose (37).

Modified-live LT vaccines have been associated with a variety of adverse effects including spread of vaccine virus to nonvaccinates (6, 35, 77, 161), insufficient attenuation, production of latently infected carriers (8), and increased virulence as a result of *in vivo* (bird-to-bird) passage (72). Laryngotracheitis vaccine viruses have been shown to spread readily from vaccinated to nonvaccinated chickens (6, 35, 77, 161). Such spread should be avoided, as spread to nonvaccinates results in *in vivo* (bird-to-bird) passage and possible reversion of vaccine virus to virulence (72). Alternatively, vaccine virus may result in disease in unvaccinated chickens due to insufficient attenuation. Spread of vaccine viruses may be prevented by biosecurity measures that prevent flock-to-flock spread, and by using vaccination methods that ensure simultaneous infection with LT vaccine virus of all susceptible birds on a farm.

Guy *et al*. (70, 71, 72) provided evidence indicating involvement of modified-live LT vaccine viruses in field outbreaks. They suggested that modified-live LT vaccine viruses increase in virulence as a result of vaccine virus spread and *in vivo* (bird-to-bird) passage. In studies comparing six modified-live LT vaccine viruses and field LTV isolates, vaccine viruses were shown to be indistinguishable from field isolates based on DNA-restriction endonuclease analyses (70), but the virulence of all vaccine viruses was low compared with field isolates (71). Two modified-live vaccine viruses, a chicken embryo-origin (CEO) virus and a tissue culture-origin (TCO) virus, were sequentially passaged in specific-pathogen-free chickens to determine whether virulence of vaccine viruses could increase after sequential *in vivo* passage (72). Sequential passage of modified-live LT vaccine viruses resulted in increased virulence of the CEO virus but not the TCO virus. After 10 sequential passages in chickens, the CEO virus possessed virulence comparable to that of a highly virulent reference strain (Illinois N71851 strain, ATCC VR-783). Guy *et al*. (72) suggested that increased virulence of modified-live LT vaccine viruses may occur in field situations as a result of drinking-water vaccination and poor biosecurity, conditions that allow uncontrolled spread of vaccine viruses to nonvaccinates, and sequential *in vivo* passage of vaccine viruses.

Inactivated Vaccines

Experimental vaccines have been prepared from inactivated whole LTV (12, 52) or affinity-purified preparations of LTV glycoproteins (193). These vaccines have been shown to stimulate immune responses in chickens and varying degrees of protection to LTV challenge. Practical field use of these types of vaccines, however, is unlikely due to high cost of preparation and delivery.

Vaccines Based on Recombinant DNA Technology

A variety of strategies for development of LT vaccines based on recombinant DNA technology recently were reviewed by Bagust and Johnson (10). They suggested that this type of vaccine could be used in conjunction with quarantine and hygiene measures for the development of regional LTV eradication programs.

Vaccines based on recombinant DNA technology have been developed for LTV control. These include recombinant, live LTV vaccines constructed by insertion of LTV genes into virus-vectors, and by alteration or deletion of viral genes. Recombinant, virus-vectored vaccines for immunization of chickens against LTV have been produced and evaluated (47, 159, 175). Saif *et al*. (159) evaluated the protective efficacy of a recombinant herpesvirus of turkeys (HVT) containing LTV genes; they reported that this recombinant vaccine produced protection against LTV challenge similar to that induced by modified-live virus vaccines. Tong *et al*. (175) constructed a recombinant fowlpox virus containing LTV gB gene; they reported that this vaccine produced protection against LTV challenge similar to that induced by modified-live vaccines. Davison *et al*. (47) evaluated a fowlpox virus recombinant containing LTV gB and UL-32 genes; they reported that this vaccine provided adequate immunity against LTV challenge.

Recombinant, live LTV vaccines have been constructed by alteration or deletion of viral genes, producing LTV mutants that lack genes coding for virulence factors. These mutants may then be capable of inducing protective immunity, without the capability to induce disease. Several LTV deletion mutants have been developed for potential use in the control of LTV (57, 58, 69, 124, 137, 163, 181, 182). Guo *et al*. (69) described the construction of a recombinant LTV expressing the β-galactosidase

marker gene by insertion of this gene into an open reading frame of the LTV DNA. Okamura *et al*. (137) and Schnitzlein *et al*. (163) developed recombinant LTV lacking thymidine kinase, a herpesvirus virulence factor, by inserting *Lac-Z* marker genes into the thymidine kinase gene of the viral DNA. The green fluorescent protein (GFP) also has been utilized as a marker to produce recombinant LTV. Fuchs *et al*. (57) produced a UL50 gene deletion mutant expressing GFP and concluded that the LTV UL-50 gene codes for a viral dUTPase enzyme, a virulence factor that is not required for replication of the virus in the respiratory tract of birds. Laryngotracheitis virus mutants lacking the gJ (58), gG (49), and UL0 genes (181) were shown to have minor growth defects in cell culture and showed an attenuated phenotype in chickens while maintaining their immunogenicity. Deletion mutant vaccines composed of gene deletions in the thymidine kinase gene (137, 163), UL0 gene (181), and gJ gene (58) are considered to be suitable candidates for vaccine use. The gJ gene mutant is of particular interest because antibodies against the gJ are present during a natural infection; therefore, the lack of this glycoprotein in the vaccine strain potentially allows identification of LTV-infected birds by detection of gJ-specific antibodies (58). Laryngotracheitis virus deletion mutants also have been used as a vector to express the H7 and H5 genes of avian influenza virus, and the application of these mutants as bivalent vaccines to induce protection against LTV and avian influenza infections has been proposed (124, 181). A variety of other LTV deletion mutant viruses have been evaluated in birds; these include LTV mutants lacking five unique open reading frames (ORF A–ORF E) (182), and mutant viruses having deletions of the UL49.5 gene that codes for gN, and the UL10 gene, which codes for the non-glycosylated membrane protein M (55, 56).

An LTV deletion mutant was constructed by deletion of adjacent genes US7 and US8 by insertion of a gene coding for green fluorescent protein; US7 and US8 genes code for gI and gE, respectively (48). This LTV deletion mutant could not be propagated in cell culture suggesting that these glycoproteins are essential for LTV replication.

Field Vaccination Protocols and Regimens

Chickens may be successfully vaccinated as early as 1 day of age (145); however, chickens less that 2 weeks of age do not respond as well as older birds (5, 40, 62). Additionally, severe reactions are more likely in younger chickens.

In chickens older than 2 wk of age, LT vaccination using modified-live vaccines or field exposure confers protection against challenge, which is partial by 3–4 days postvaccination and complete by 6–8 days (20, 61, 81). Waning of immunity has been detected as early as 8–15 wk postvaccination (83), but substantial flock immunity generally is observed for 15–20 wk after vaccination (5, 62, 139). Vaccine breaks in the field are most commonly observed after 15–20 wk postvaccination, but the value of revaccination is questionable (102). Revaccination with modified-live vaccines may be ineffectual in maintaining protection levels because the infectivity of vaccine virus may be neutralized and replication prevented by existing immunity (51, 196).

Laryngotracheitis can be well controlled in multiple-age layer flocks by vaccination with modified-live vaccines. Layer flocks generally are vaccinated twice before the onset of egg production; vaccines typically are administered by eye drop at approximately 7 weeks of age and again at approximately 15 weeks of age by eye drop, spray, or drinking water. Studies by Fulton *et al*. (59) demonstrated the importance of two vaccinations for development of protection against challenge. Two vaccinations resulted in superior protection compared with a single vaccination, regardless of route (eye drop, spray, water) and vaccine source. Vaccine application by eye-drop route was shown to provide more uniform protection following a single dose compared with spray and drinking water routes. Single dose vaccination by the drinking water route was dependent upon the proximity of birds to the water source, and some vaccines failed to provide protection when a single dose was administered by spray (59).

For intensive broiler production, the short growth cycle, all in-all out production, and a high level of biosecurity can reduce the need for prophylactic vaccination. However, vaccination of broiler flocks may be necessary when these flocks are in the vicinity of LT outbreaks or when disease has previously occurred on the farm. Under these circumstances, broiler chickens generally are vaccinated at 10–21 days of age, usually via the drinking water route.

A recombinant fowl pox virus-vectored vaccine for immunization of chickens against LTV is commercially available in the United States (47). This vaccine is used for immunization of multi-age layer flocks. It is administered by wing-web inoculation to chickens that are at least 8 weeks of age and at least 4 weeks prior to onset of egg production.

Treatment

No drug has been shown to be effective in reducing the severity of lesions or relieving disease signs. If a diagnosis of LT is obtained early in an outbreak, vaccination of unaffected birds may induce adequate protection before they become exposed.

Eradication

Eradication of LTV from intensive poultry production sites appears to be highly feasible owing to several biologic and ecologic properties of the virus, which have been reviewed in detail by Bagust and Johnson (10). These properties include the high degree of host specificity of LTV, fragility of virus infectivity outside the chicken, and antigenic stability of the LTV genome. The chicken is the primary host species and reservoir host; wildlife reservoirs are believed either to be nonexistent or of minor importance in LTV ecology. Backyard and fancier chicken flocks are likely reservoirs of LTV; thus, any eradication effort would require identification and inclusion of these birds (125). Laryngotracheitis virus strains are antigenically homogeneous; thus, a single LTV vaccine produces cross-protective immunity for all LTV strains.

Eradication of LTV will require a change in current LT vaccination practices. This will entail the replacement of conventional, modified-live vaccines in vaccination programs with vaccines produced by recombinant DNA technology; vaccines produced by this technology induce protective immunity without development of latently infected carrier chickens (10).

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Chapter 6

Influenza

D. E. Swayne and D. A. Halvorson

Introduction

The term "influenza" originally referred to epidemics of acute, rapidly spreading catarrhal fevers of humans caused by viruses in the family *Orthomyxoviridae* (142). Today, orthomyxoviruses are recognized as the cause of significant numbers of natural infections and disease, usually of the upper respiratory tract, in humans, horses, domestic pigs, and various bird species and sporadic cases of naturally occurring disease in mink and a variety of marine mammals (88,172,333). Since 2003, isolated natural cases of H5N1 high pathogenicity avian influenza (HPAI) have been reported in leopards, tigers, domestic cats, dogs, stone martins, civets, and domestic pigs, which were exposed to H5N1 HPAI-infected birds, but these avian influenza (AI) virus infections have not become endemic in these species (62,138,153, 223,260,356). Infection of domestic poultry by AI viruses typically produces syndromes ranging from asymptomatic infection to respiratory disease and drops in egg production to severe, systemic disease with near 100% mortality (80). The latter form of disease is the result of infection by high pathogenicity or highly pathogenic (HP) AI viruses. Disease is usually absent with AI virus infection in most free-flying waterfowl species.

For more detail, see reviews on HPAI (308), outbreaks of AI in the 1990s (212), immunology of AI (278), AI in different bird species (10), epidemiology and control (8), and evolution and ecology of AI viruses (283,333).

Definitions and Synonyms

Avian influenza initially was recognized as a highly lethal, systemic disease (i.e., highly pathogenic or highly virulent AI). From the late 1870s to 1981, HPAI was known by various names including fowl plague (most common), fowl pest, peste aviaire, Geflugelpest, typhus exudatious gallinarium, Brunswick bird plague, Brunswick disease, fowl disease, and fowl or bird grippe (272,273). In 1981 at the First International Symposium on Avian Influenza, the terminology "highly pathogenic avian influenza," was adopted as the official designation for the highly virulent form of AI (21). "High pathogenicity" is an equivalent grammatical variant of "highly pathogenic" and can be used interchangeably.

Milder forms of AI were first recognized in various domestic poultry species between 1949 and the mid-1960s and have been termed low pathogenic, pathogenic, non-highly pathogenic and low pathogenicity AI (6,83,238). Their impact on poultry production and trade has been much less severe than with HPAI. At the Fifth International Symposium on Avian Influenza in 2002, the terminology "low pathogenicity (LP)" was adopted as the official designation for low virulence AI (i.e., any AI that does not meet the criteria for HPAI (95)).

The World Organization for Animal Health (Office International des Epizooties [OIE]) is an intergovernmental organization charged by the World Trade Organization to set the sanitary and health standards for animal diseases. The OIE uses the designation of notifiable AI (HP notifiable AI [HPNAI] and LP notifiable AI [LPNAI] for international animal health regulatory purposes (9,199). HPNAI encompasses all HPAI while LPNAI encompasses only H5 and H7 LPAI. Prior to 2004, OIE Terrestrial Animal Health Code covered only HPAI, which was on the OIE's most serious disease list, List A; however, since then, the List A and B system has been eliminated.

Economic Significance

Economic losses from AI have varied depending on the strain of virus, species of bird infected, number of farms involved, control methods used, and the speed of implementation of control or eradication strategies. In most developed countries, HP and LPAI have not been endemic diseases in the commercial poultry industries. Most outbreaks and economic losses have occurred from epidemics of HP or LPAI in commercially raised poultry, predominately chickens and turkeys. In some developing countries LPAI has been endemic in commercially raised poultry—especially viruses of the H9N2 subtype in the 1990s. In some developed countries, LPAI has been endemic in backyard and live poultry market (LPM) systems that serve ethnic populations of large metropolitan areas. Since 2003, H5N1 HPAI has become endemic in village poultry, especially domestic ducks in some parts of the world.

Generally, the most accurate reports on losses have resulted from HPAI eradication programs (Table 6.1). Direct losses in HPAI outbreaks have included depopulation and disposal costs, high morbidity and mortality losses, cleaning and disinfection, quarantine and surveillance costs, and indemnities paid for the birds. However, indirect costs such as uncompensated losses to the poultry industry including temporary or permanent loss in poultry exports, income lost by farmers and communities during the production down time, increased consumer costs from reduced supply of poultry products, and losses from decreases in consumer purchases can easily escalate losses by 5–10 fold. The economic costs for eradication of HPAI have varied greatly, but eradication costs have been very high and appear to be proportional to the number of birds that died and were culled

Table 6.1. Examples of economic losses from HPAI and LPAI epidemics as reported in US dollars.

Year	Outbreak	Birds dead or culled	Cost item	Original cost	Cost in 2007 $US	Cost/farm in 2007 $US	Reference
HPAI							
1924–25	USA—Fowl Plague	unknown	Direct losses	$1M	$12.2M	—	(273)
1983–84	USA—H5N2 HPAI	17M (449 farms)	USDA eradication	$63M	$126M	$280,000	(94,161)
			Non-indemnified industry losses	$15M	$30M	$66,500	
			Increased customer costs	$349M	$700M	$1.5M	
1985	Australia—H7N7 HPAI	238,518 (1 farm)	Eradication cost	$1.4M	$2.7M	$2.7M	(68)
1999–2000	Italy—H7N1 HPAI	13M (413 farms)	Compensation	$100M	$121M	$298,000	(298)
			Indirect costs	$500M	$605M	$1.5M	
1997	Hong Kong—H5N1 HPAI	1.5 M	Eradication	$13M	$17M	—	(298)
Late 2003–mid-2005	Asia—H5N1 HPAI	220M	Losses to the poultry industries	>$10B	>$10B	—	(353)
LPAI							
1978	Minnesota USA—various LPAI	141 farms	Losses to the poultry industries	$5M	$16M	$113,000	(110)
1995	Minnesota USA—H9N2 LPAI	178 farms	Losses to the poultry industries	$6M	$8.2M	$46,000	(110)
1978–1995	Minnesota USA—various LPAI	1058 farms	Losses to the poultry industries	$22M		$21,000	(110)
1995	Utah USA—H7N3 LPAI	2M (60 farms)	Losses to the poultry industries	$2M	$2.7M	$45,000	(110,208)
2002	Virginia USA—H7N2 LPAI	4.7M (197 farms)	USDA eradication	$81M	$94M	$477,000	(3)
			Losses to the poultry industries	$130M	$150M	$761,000	
			State government	$1M	$1.2M	$6000	

(Table 6.1). However, in the 1983–84 USA H5N2 HPAI epidemic, the projected cost of not implementing an eradication program was $500 million for losses to poultry farmers and $5.5 billion in increased customer costs (161).

Low pathogenicity AI outbreaks have caused significant economic losses for producers of chickens, turkeys and ducks, especially when accompanied by secondary bacterial or viral pathogens, but accurate documentation of such costs are generally not available. In general, losses have been less than with HPAI outbreaks because infected flocks have typically been eliminated through a controlled marketing program, the mortality rates have been lower, no federal eradication costs were incurred, and national and international trade usually have not been disrupted (Table 6.1). Losses from LPAI epidemics include mortality losses, condemnations at slaughter, medication against secondary bacteria, cleaning and disinfection, and delayed placements of new birds. Poorly documented but more costly have been the endemic H9N2 LPAI poultry infections in much of Asia and the Middle East and H5N2 LPAI poultry infections in Mexico and Central America. Since LPAI is usually not dealt with by traditional stamping-out programs, the costs of LPAI are unknown. However, when a stamping-out program was undertaken in the Virginia 2002 H7N2 LPAI outbreak, the eradication program had similar costs as previous HPAI outbreaks (Table 6.1).

Public Health Significance

In general, influenza viruses exhibit host species adaptation with transmission occurring most frequently and with ease between individuals of the same species; occasionally interspecies transmission to closely related species occurs (283). On rare occasions, AI viruses have exhibited interspecies transmissibility to humans (80). Although rare, AI viruses or their genes have been transferred to humans: 1) transfer of complete AI viruses (*in toto*) with individual sporadic infections, and 2) appearance of individual AI viral gene segments in pandemic human influenza viruses (i.e., reassortment of gene segments).

Transfer of Complete AI Virus

Sporadic cases of transmission of whole AI viruses to humans have been documented (42,283). However, such cases have been rare compared to the hundreds of millions of human infections by H1N1 and H3N2 human-adapted influenza viruses that occur each year. Variations in cell receptor specificity on the respiratory tract epithelium may account for some of the differences in influenza virus transmission and efficient replication. AI viruses have preferential binding to N-acetylneuraminic acid-α2, 3-galactose linkage on sialoligosaccharide (α2,3 linkage) receptors, and human influenza viruses preferentially bind to N-acetylneuraminic acid-α2,6-galactose linkage on sialoligosac-

Table 6.2. Listing of confirmed human cases of infection with avian influenza viruses (modified from (214,346)).

Year	Virus	Location	Symptoms	Exposure	Cases	Deaths	Reference
1959	H7N7 HPAI	USA	Hepatitis	Unknown	1	0	(72)
1977	H7N7 HPAI	Australia	Conjunctivitis	Laboratory accidental exposure	1	0	(314)
1978–79	H7N7 LPAI	USA	Conjunctivitis	Seals with respiratory disease	Not reported	0	(335,337)
1996	H7N7 LPAI	United Kingdom	Conjunctivitis	Tending domestic ducks that mixed with wild ducks on a pond	1	0	(23,155)
1998, 2003	H9N2 LPAI	Hong Kong and Mainland China	3 = respiratory symptoms; 5 = influenza-like illness	1 = contact with live poultry in LPM, 7 = not reported	8	0	(105,210,346)
2002–03	H7N2 LPAI	USA	1 = asymptomatic, 1 = respiratory symptoms	1 = outbreak crew, 1 = unknown	2	0	(56,57)
2003	H7N7 HPAI	Netherlands	Conjunctivitis > influenza-like illness > other symptoms	Depopulation crews, poultry workers and farmers from poultry H7N7 HPAI outbreak	89	1	(150)
2004	H7N3 HPAI	Canada	Conjunctivitis, coryza and headache	Depopulation crews for poultry H7N3 HPAI outbreak	2	0	(323)
1997–2006	H5N1 HPAI	Asia, Africa[1]	Respiratory symptoms < gastrointestinal	Exposure to live or dead infected poultry in LPM or villages	348	207	(187,209,347)
				Total	452	208	

[1]Reflect the cases as of 08/31/2007

charide (α2,6 linkage) receptors (128). Avian respiratory epithelium has predominantly α2,3 linkage while human respiratory epithelium has predominately α2,6 linkage (128). Although the human respiratory tract does have α2,3 receptors on cells deep in the respiratory tract—non-ciliated cuboidal bronchiolar and alveolar type II cells—which provide a site for potential AI virus replication (243), but the deep location of these receptors make exposure unlikely and the resulting human AI virus infections rare. In addition, other AI viral genes—e.g., polymerase complex—may confer inefficient replication in humans (243).

Nine incidents of limited natural human infection with *in toto* transfer of AI viruses have been reported over the past 50 years (Table 6.2). Most consistently, human AI cases presented with conjunctivitis (predominantly H7 cases), respiratory illness (predominantly H5 cases) or flu-like symptoms, but some cases presented with atypical signalment such as gastrointestinal symptoms (346). Six incidents involved 15 cases while the 2003 Netherlands H7N7 and 1997–2006 H5N1 HPAI viruses account for 207 of the 208 human infections and the H5N1 HPAI virus for 437 of 452 fatalities (Table 6.2). The H5N1 HPAI cases have occurred across a broad geographic area involving cases in multiple countries including Azerbaijan, Cambodia, Djibouti, Hong Kong, and Mainland China, Egypt, Indonesia, Iraq, Thailand, Turkey, and Vietnam, but the infecting viruses have all been direct descendants of a single AI H5 hemagglutinin gene. Most of the H5N1 HPAI cases have been linked to direct exposure to H5N1 HPAI virus infected live or dead poultry within LPM or villages (213,245). Two cases are exceptions with presumed exposure through consumption of raw duck blood and organs, and defeathering H5N1 infected dead swans (345,348). Finally, in a study in Cambodia, villagers with close frequent contact to H5N1 HPAI virus infected poultry did not become infected which suggests transmission potential from poultry to individual humans is low (331). The cases listed in Table 6.2 and experimental data in animal models and humans suggest that some AI viruses, such as the H5N1 HPAI virus, have a greater potential to infect humans than other HPAI viruses and most LPAI viruses (33,77).

Transfer of AI Virus Gene Segments

Wild waterfowl and other aquatic birds are the primordial reservoir of all influenza viral genes (333). Although the probability of an AI virus entering the human population, reassorting and establishing a new lineage of human influenza virus has been extremely rare, which is consistent with the long time spans between the emergence of new human pandemic influenza viruses (23). For example, analysis of nucleotide sequence data has determined that the 1957 (H2N2) and 1968 (H3N2) human pandemic influenza viruses resulted from the reassortment of three (HA, NA, and PB1) and two (HA and PB1) AI viral genes with five and six human influenza viral genes, respectively (135,222,228,229). In theory, swine have been proposed as the "mixing" vessel for co-infection by influenza viruses from birds

and mammals with development of "new" strains (reassortants) having the ability to infect people and other mammals (230). However, in view of the human H5N1 HPAI virus infection and lack of H2N2 and H3N2 virus infections in pigs during 1957 and 1968 pandemics, reassortment of gene segments between a human adapted influenza virus and AI virus could have occurred in dually infected humans. Some recent evidence suggests that the 1918 pandemic virus was not derived by reassortment but arose by adaptation of a complete AI virus (313).

History

The history of avian influenza can be divided into three general periods: 1) early reports of HPAI, 2) recognition of less severe AI disease (LPAI) in domestic poultry, and 3) identification of AI viruses from asymptomatic wild bird reservoirs. Avian influenza was first reported as HPAI ("fowl plague") in 1878 by Perroncito in Italy (273). Initially, the disease was confused with the acute septicemic form of fowl cholera until 1880 when Rivolto and Delprato differentiated the two based on clinical and pathological features. In 1901, Centanni and Savonuzzi determined the cause was a filterable agent, but the virus was not identified nor classified as an influenza virus until 1955 (227,228,273).

In 1894, a severe outbreak of HPAI occurred in northern Italy and was disseminated via transport of chickens to eastern Austria, Germany, Belgium, and France (176,272). HPAI was spread throughout Germany as the result of the 1901 Brunswick Fowl Exposition (272). By the early part of the twentieth century, HPAI was reported in Switzerland, Romania, Russia, Netherlands, Hungary, Great Britain, Egypt, China, Japan, Brazil, and Argentina (152,182,273). By the mid-twentieth century, HPAI had been diagnosed in most of Europe, Russia, North Africa, Middle East, Asia, South America, and North America (82). In many parts of Europe, HPAI was endemic until the mid-1930s (11). HPAI was reported in the United States in 1924–1925 and 1929 (273). The HPAI outbreak began in 1924 with severe losses in the LPM system of New York and later New Jersey and Philadelphia, Pennsylvania (273). In 1925, infected farms or markets were identified in Connecticut, West Virginia, Indiana, Illinois, Michigan, and Missouri. The 1929 outbreak involved a few flocks in New Jersey (273). Quarantine, depopulation, cleaning, and disinfection were used to eradicate HPAI from the United States.

The outbreaks of HPAI between 1901 and the mid-1950s involved isolates that today have been classified as H7N1 and H7N7 subtypes (see Strain Classification) (80). However, an outbreak during 1959 in chickens of Scotland and during 1961 in common terns (*Sterna hirundo*) of South Africa involved new subtypes of AI viruses, H5N1 and H5N3, respectively (308). This led to the establishment of the erroneous dogma that all H5 and H7 AI viruses were highly pathogenic. Table 6.3 lists the summaries of HPAI outbreaks between 1955 and 2007. Of the 26 epidemics, 23 have involved domesticated poultry, principally chickens and turkeys, one involved exclusively wild birds (i.e., common terns) and one involved both domestic poultry, including ducks and geese, and wild birds. Details on individual outbreaks can be obtained from the references listed in Table 6.3.

Table 6.3. Twenty-six documented epidemics of HP AI since discovery of AI virus as cause of fowl plague in 1955, modified from (11,298,308).

Dates	Prototype AI Virus	Subtype	Number Affected with High Mortality or Were Depopulated(a)	Specific References
1959	A/chicken/Scotland/59	H5N1	2 flocks of chickens (*Gallus gallus domesticus*)—total number of birds affected not reported	215) (D.J. Alexander, (personal communication, 2000)
1961	A/tern/South Africa/61	H5N3	1300 common terns (*Sterna hirundo*)	(35)
1963	A/turkey/England/63	H7N3	29,000 breeder turkeys (*Meleagridis gallopavo*)	(343)
1966	A/turkey/Ontario/7732/66	H5N9	8,100 breeder turkeys	(160)
1975-76	A/chicken/Victoria/75 or A/chicken/Victoria/76	H7N7	25,000 laying chickens, 17,000 broilers, and 16,000 ducks (*Anas platyrhyncos*)	(18,322)
1979	A/chicken/Germany/79	H7N7	Unknown (formerly East Germany)	(11)
1979	A/turkey/England/199/79	H7N7	3 commercial farms of turkeys—total number of birds affected not reported	(4,16)
1983–84	A/chicken/Pennsylvania/1370/83	H5N2	17 million birds in 452 flocks; most were chickens or turkeys, a few chukar partridges (*Alectoris chukar*) and guinea fowl (*Numida meleagris*)	(80,84,325)
1983	A/turkey/Ireland/1378/83	H5N8	800 meat turkeys died on original farm; 8,640 turkeys, 28,020 chickens and 270,000 ducks were depopulated on original and 2 adjacent farms	(7,178)
1985	A/chicken/Victoria/185	H7N7	24,000 broiler breeders, 27,000 laying chickens, 69,000 broilers and 118,518 unspecified-type of chickens	(24,68)
1991	A/turkey/England/50–92/91	H5N1	8000 turkeys	(14)
1992	A/chicken/Victoria/192	H7N3	12,700 broiler breeders, 5,700 ducks	(234,344)
1994	A/chicken/Queensland/477/94	H7N3	22,000 laying chickens	(344)

Table 6.3. Twenty-six documented epidemics of HP AI since discovery of AI virus as cause of fowl plague in 1955, modified from (11,298,308). (*continued*)

Dates	Prototype AI Virus	Subtype	Number Affected with High Mortality or Were Depopulated(a)	Specific References
1994–95	A/chicken/Puebla/8623–607/94 A/chicken/Queretaro/14588–19/95	H5N2	Chickens(b)	(80,330)
1994–95, 2004	A/chicken/Pakistan/447/95 A/chicken/Pakistan/1369-CR2/95	H7N3	Two incursions: 1) 3.2 million broilers and broiler breeder chickens (northern part of country—1994–5), 2) 2.52 million layers (Karachi—2004) (c)	(80,190)
1996–2007	A/goose/Guangdong/1/1996 A/chicken/Hong Kong/220/97	H5N1	Over 220 million birds dead or culled, mostly chickens, but also ducks, geese, Japanese quail and some wildbirds(d)	(91,248,249)
1997	A/chicken/New South Wales/ 1651/97	H7N4	128,000 broiler breeders, 33,000 broilers, 261 emu (*Dromaius novaehollandiae*)	(213)
1997	A/chicken/Italy/330/97	H5N2	2116 chickens, 1501 turkeys, 731 guinea fowl, 2322 ducks, 204 quail (species unknown), 45 pigeons (*Columbia livia*), 45 geese (species unknown) and 1 pheasant (species unknown)	(51)
1999–2000	A/turkey/Italy/4580/99	H7N1	413 farms—8.1 million laying chickens; 2.7 million meat and breeder turkeys; 2.4 million broiler breeders and broilers; 247,000 guinea fowl; 260,000 quail, ducks and pheasants; 1,737 backyard poultry and 387 ostriches	(49)
2002	A/chicken/Chile/184240–1/2002	H7N3	Two farm, multiple houses; 617,800 broiler breeders, unspecified number turkey breeders (2 houses)	(226)
2003	A/chicken/Netherlands/ 621557/ 2003	H7N7	255 infected flocks, 1381 commercial and 16,521 backyard/smallholder flocks depopulated. 30 million died or depopulated—majority were chickens	(85)
2004	A/Chicken/Canada/AVFV2/04	H7N3	42 commercial and 11 backyard flocks infected (1.2 million poultry)—approximately 16 million commercial poultry depopulated, most were chickens	(123,195)
2004	A/chicken/Texas/298313/2004	H5N2	1 non-commercial farm and 2 live poultry markets, 6600 chickens	(168,197)
2004, 2006	A/ostrich/South Africa/2004	H5N2	2004—11 ostrich farms with depopulation of 23,625 ostriches and 3,550 other poultry (chickens, turkeys, geese, ducks and pigeons); 2006—7342 ostriches dead or culled	(196,200,201)
2005	A/chicken/North Korea/1/2005	H7N7	3 farms, 218,882 chickens culled; number dead not reported.	(197,198)
2007	Not available	H7N3	1 farm, 48,560 broiler breeders culled; 540 roosters died	(201a)

(a) Most outbreaks were controlled by "stamping out" or depopulation policies for infected and/or exposed populations of birds. Chickens, turkeys and birds in the order *Galliformes* had clinical signs and mortality patterns consistent with HPAI while ducks, geese and other birds lacked or had low mortality rates or infrequent presence of clinical signs.

(b) "Stamping-out" policy was not used for control. The AI outbreak had concurrent circulation of LP and HPAI virus strains. However, HPAI virus strains were present only from late 1994 to mid-1995. Estimates of number of birds infected with HP AI strains are unavailable but 360 commercial chicken flocks were "depopulated" for AI in 1995 through controlled marketing.

(c) "Stamping-out" policy was not used for control. Surveillance, quarantine, vaccination and controlled marketing were used as the control strategy. The numbers affected are crude estimates from 2 separate time periods of clinical disease outbreaks, but the virus lineage is the same between the outbreaks.

(d) The H5 and N1 gene lineages have been maintained among the HPAI viruses from outbreaks in various Asian, African and European countries (1996–2007). The six internal gene segments have undergone reassortment. The initial H5N1 HPAI outbreaks were reported in China (1996) with three incursions in Hong Kong (1997, 2001 and 2002). This was followed by regional extension with outbreaks in 2003–2005 within Southeast Asia (South Korea, Vietnam, Japan, Indonesia, Thailand, Cambodia, Laos, China and Malaysia). In mid-late 2005, outbreaks occurred in both wild birds and poultry in central Asia with extension to Eastern Europe and Middle East by fall of 2005. In 2006, outbreaks were reported in Africa. Initially, chickens were the main species affected with disease and death, but in many of the outbreaks, domestic ducks have emerged to be a major species in maintenance and epidemiology of the viruses. Various wild birds have succumbed to infections.

Milder diseases caused by AI viruses were recognized beginning in the middle of the twentieth century (82). Today, these AI viruses are termed low pathogenicity AI (LPAI) (see "Strain Classification" and "Pathogenicity"). The oldest existing LPAI virus, the Dinter strain from Germany, was isolated from chickens in 1949, but its identification as AI virus was not until 1960 (A/chicken/Germany/49 [H10N7]). Similarly, LPAI viruses were isolated from domestic ducks with respiratory disease between 1953–1963 in Canada, Czechoslovakia, England, and the Ukraine (82). Low pathogenicity avian influenza viruses emerged as a cause of respiratory disease and drops in egg production in turkeys in Canada and the United States during the early 1960s. Of notable significance was the identification of LPAI viruses of the H5 subtype in Canada during 1966 and the United States (Wisconsin) during 1968 (5,82,258). In 1971, a turkey flock in Oregon experienced mild respiratory disease with diarrhea, and an H7N3 AI virus was isolated (31). Since 1971, numerous H5 and H7 LPAI viruses have been isolated and characterized, thus dispelling the myth that subtypes H5 and H7 equate with HP (5,80,119).

Many AI viruses have been isolated from asymptomatic infections in wild aquatic birds. Initially, serologic surveys of migratory waterfowl showed evidence of infection by AI viruses (81). During 1972, AI viruses were isolated from migratory ducks as part of a Newcastle disease virus surveillance program (254) and in Australia from a pelagic seabird (shearwater) (75). Since then, large surveys have demonstrated that healthy wild aquatic birds, principally in the orders *Anseriformes* and *Charadriiformes*, have been asymptomatic reservoirs of AI viruses (265). AI viruses from wild birds have been mostly LP for domestic poultry. However, a few HPAI viruses have been isolated from wild birds: 1) during an epidemic with high mortality in common terns (A/tern/South Africa/61 [H5N3]); 2) single isolations of A/finch/Germany/72 (H7N1), A/gull/Germany/79 (H7N7) and A/Peregrine Falcon/UAE/2384/98 (H7N3); and 3) during the H5N1 HPAI outbreak in Asia, Europe and Africa with multiple isolations from wild birds (7,61,170,174).

Since AI is an international concern, global interest in AI resulted in the convening of international symposia in 1981, 1986, 1992, 1997, 2002 and 2006 to deal with AI issues (22,78,79,287, 307,309). Because influenza is an international problem, solutions will require international efforts and cooperation (80).

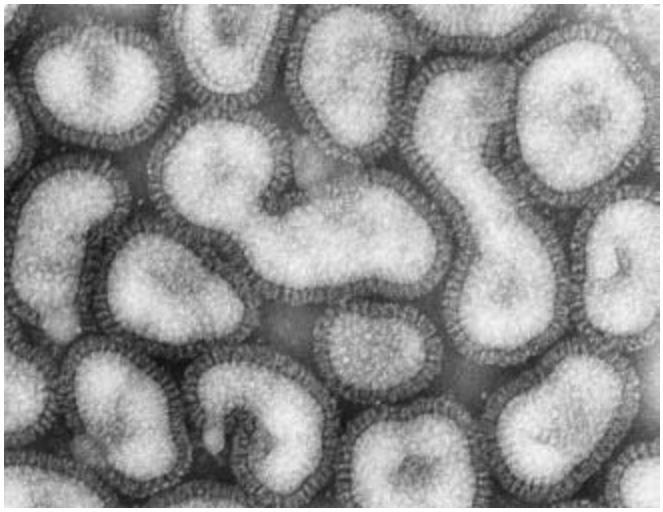
Etiology

Classification

Avian influenza viruses are classified in the family *Orthomyxoviridae*, genus *Influenzavirus A* (67).

Morphology

Virions are typically spherical to pleomorphic but can be filamentous (67). Individual virions range in diameter from 80–120 nm, but the filamentous forms can have lengths up to several hundred nm (Fig. 6.1) (67). The surface is covered by two types of glycoprotein projections (10–14 nm in length and 4–6 nm in diameter): 1) rod-shaped trimers of hemagglutinin (HA), and 2) mushroom-shaped tetramers of neuraminidase (NA). Virus buoyant density is 1.19 g/cm^3 in aqueous sucrose and single virion molecular weight (Mr) is 250 × 10^6 (67).



6.1. Purified A/WSN/33 influenza A virus. Negative stain with 2% phosphotungstic acid. ×282,100. (Gopal Murti)

The nucleocapsid is helical. The viral genome is composed of eight segments of single-stranded, negative-sense RNA that code for 10 proteins. Their size and function are listed in Table 6.4. Eight proteins are constituents of the virus (HA, NA, NP, M1, M2, PB1, PB2, and HA), and the two nonstructural proteins (NS1 and NS2) are located in the host cell cytoplasm. Recently, NS2 has been shown to also be a minor constituent of virions (156).

Chemical Composition

Influenza virions are composed of 0.8–1.0% RNA, 5–8% carbohydrate, 20% lipid and 70% protein (156). The carbohydrates are contained within glycolipids and glycoproteins and include galactose, mannose, fucose, and glucosamine (144). Ribose is contained in the RNA genome. Lipids are present in the viral envelop and are derived from the host cell. Most of the lipids are phospholipids, but small amounts of cholesterol and glycolipid are present. The viral genome specifies the proteins and their potential glycosylation sites.

Virus Replication

The stages of virus replication have been reported by various investigators in great detail (156,203) or in brief (67,80). In brief, AI virus HA adsorbs to host cell receptors containing sialic acid bound to glycoproteins, thus initiating receptor-mediated endocytosis. In the endosomes, low-pH-dependent fusion occurs via HA-mediated fusion of viral envelope with the endosome membrane. Proteolytic cleavage of HA into HA1 and HA2 is an essential prerequisite for fusion and infectivity. The viral nucleocapsids are transported to the nucleus where viral transcriptase complex synthesizes mRNA. Transcription is initiated with 10–13 nucleotide RNA fragments generated from host heterogenous nuclear RNA via viral endonuclease activity of PB2. Six monocistronic mRNAs

Table 6.4. Gene and protein information on *Influenzavirus A* (67,157,203).

Genome		Proteins Coded				
Segment	Length (nucleotides[a])	Name	Length (aa)	App. No. molecules/ virion	Type	Function
1	2341	PB1	759	30–60	Polymerase complex	Transcriptase
2	2341	PB2	757	30–60	Polymerase complex	Endonuclease
3	2233	PA	716	30–60	Polymerase complex	1. Viral RNA replication. 2. Proteolytic activity
4	1778	Hemagglutinin (HA)	566	500	Integrated type I membrane glycoprotein	1. Virus attachment to sialyloligosaccharide cell receptors including hemagglutinating activity. 2. Envelop fusion. 3. Antibody-mediated viral neutralization
5	1565	Nucleoprotein (NP)	498	1000	Major structural protein—associated with viral RNA segments	1. Cytoplasmic to nuclear (NP) protein—transport of viral RNP. 2. Necessary for full length vRNA synthesis. 3. Antigen target for cytotoxic T lymphocytes
6	1413	Neuraminidase (NA)	454	100	Integrated type II membrane glycoprotein	1. Cell receptor-destroying enzyme (sialic acid residues) that causes virus elution. 2. Antibody-mediated virus neutralization restricts virus spread
7	1027	Matrix 1 (M1)	252	3000	Non-glycosylated structural protein beneath viral envelope	Most abundant protein—role in virus budding
		Matrix 2 (M2)	97	20–60	Integrated type III glycosylated membrane protein	Ion channel
8	890	Non-structural 1 (NS1)	230	—	RNA binding protein	1. Inhibit processing of cellular mRNA. 2. Enhance of cytoplasmic translation of viral mRNA. 3. Possible inhibition of interferon pathways
		Non-structural 2 (NS2)	121	130–200	Nuclear export protein	Nuclear export of viral RNP

[a]Number of nucleotides based on human influenza strain A/PR/8/34 (H1N1)

are produced in the nucleus and transported to the cytoplasm for translation into HA, NA, NP, PB1, PB2, and PA proteins. The mRNA of NS and M gene segments undergo splicing with each producing two mRNAs, which are translated into NS1, NS2, M1, and M2 proteins. The HA and NA proteins are glycosylated in the rough endoplasmic reticulum, trimmed in the Golgi and transported to the surface where they are embedded in the plasma membrane. The eight viral gene segments along with internal viral proteins (NP, PB1, PB2, PA, and M2) assemble and migrate to areas of the plasma membrane containing the integrated HA, NA, and M2 proteins. The M1 protein promotes close association with the plasma membrane and budding of the virions.

Susceptibility to Chemical and Physical Agents

Avian influenza viruses are relatively unstable in the environment. Physical factors such as heat, extremes of pH, hypertonic conditions, and dryness can inactivate AI viruses. Because AI viruses have lipid envelopes, they are inactivated by organic solvents and detergents, such as sodium desoxycholate and sodium dodecylsulfate. In the presence of organic matter, AI virus can be destroyed by chemical inactivants such as aldehydes (formaldehyde or glutaraldehyde), beta-propiolactone, and binary ethylenimine. After removal of organic matter, chemical disinfectants such as phenolics, ammonium ions (including quaternary ammonium disinfectants), oxidizing agents (such as sodium hypochlorite), dilute acids, and hydroxylamine can destroy AI viruses (99,165).

Laboratory Situation

The AI viruses are relatively stable in protein-containing solutions, but long-term storage should be at –70°C or following lyophilization. Egg grown virus can be maintained for several

weeks at 4°C without loss of infectivity, but hemagglutinating and NA activities can be maintained even longer even when the virus is no longer infectious. Inactivation with retention of hemagglutinating and NA activities can be achieved with various concentrations of formalin, binary ethylenimine, and beta-propiolactone (143). These compounds have been used as inactivants in vaccine production. Most commonly used detergents and disinfectants (such as phenolics, quaternary ammonium surfactant compounds, and sodium hypochlorite) inactivate AI viruses.

Field Situation

Influenza viruses are protected by organic material such as nasal secretions or feces, which increase resistance to physical and chemical inactivation (80). Cool and moist conditions favor long survival of AI viruses in the environment. AI viruses have been viable in liquid manure for 105 days in the winter and in feces for 30–35 days at 4°C and for 7 days at 20°C (28,94,342). Recent work on the H5N1 HPAI virus in Thailand indicated the virus could survive 4 days in chicken feces held at 25–32°C in the shade (261). In water at 28°C, the concentration of A/whooping swan/Mongolia/244/05 (H5N1) (Mongolia/05) and A/duck meat/Anyang/01 (H5N1) (Anyang/01) were reduced by 1 log in 4 and 5 days, respectively, and no virus was detected after 26 and 30 days, respectively. However, at 17°C, Mongolia/2005 and Anyang/2001 virus could persist until 158 and 94 days, respectively (38). The H5N1 HPAI virus had shorter environmental survival times compared to LPAI viruses obtained from wild waterfowl.

Proper inactivation and elimination of AI viruses shed in the environment is essential in the control of field infection and can be accomplished through integrated approaches including heating of buildings to 90–100°F for one week, thorough removal and proper disposal of manure and litter, cleaning and disinfecting of buildings and equipment, and allowing a 2–3 week vacancy period before restocking (106). Virus in manure and litter must be inactivated or disposed of by burial, composting, or incineration. Composting was effective at killing HPAI viruses within poultry carcasses in less than 10 days (237). Effective disinfectants against AI viruses on clean surfaces include 5.25% sodium hypochlorite, 2% sodium hydroxide (lye), phenolic compounds, acidified ionophor compounds, chlorine dioxide disinfectants, strong oxidizing agents, and 4% sodium carbonate/0.1% sodium silicate (65). However, organic material must be removed before disinfectants can work properly.

Pasteurization and cooking are effective means of inactivating AI viruses. USDA standard cooking times for poultry meat, which achieves an internal temperature of 165°F, and pasteurization (55.6–63.3°C, 210–372s) are adequate to kill AI viruses (286,290,315).

Strain Classification

Antigenicity

Influenza viruses are classified to genus ("type") based on serologic reactions of the internal proteins, principally NP and M1 proteins. This is typically done by an immunoprecipitation test (i.e., agar gel immunodiffusion test [AGID]) (302). All AI viruses are *Influenzavirus A* or type A. *Influenzaviruses B* and *C* (i.e., types B and C) occur in humans and rarely in seals and pigs, but neither have been isolated from birds.

Table 6.5. Nomenclature for *Influenzavirus A* subtyping (80,96,137,225,349).

Hemagglutinin		Neuraminidase	
1980–Present	**Previous**	**1980–Present**	**Previous**
H1	H0, H1, Hsw1	N1	N1
H2	H2	N2	N2
H3	H3, Heq2, Hav7	N3	Nav2, Nav3
H4	Hav4	N4	Nav4
H5	Hav5	N5	Hav5
H6	Hav6	N6	Nav1
H7	Hav1,Heq1	N7	Neq1
H8	Hav8	N8	Neq2
H9	Hav9	N9	Nav6
H10	Hav2		
H11	Hav3		
H12	Hav10		
H13	Hav11		
H14	—		
H15	—		
H16	—		

Influenzavirus A is further subtyped based on serologic reactions of the HA and NA surface glycoproteins. Sixteen subtypes of HA and nine subtypes of NA are recognized (Table 6.5). Serologic subtyping of HA is done by the hemagglutinin inhibition (HI) test and subtyping of neuraminidase by neuraminidase inhibition (NI) test (96,302). Most combinations of the 16 HA and 9 NA AI virus subtypes have been reported in domestic and wild birds, but distribution varies by year, geographic location, and host species. Since 1980, subtyping of HA and NA has been standardized for all type A influenza viruses from birds, pigs, horses, and humans (Table 6.5) (349). Prior to 1980, subtypes of HA and NA were classified according to the species of origin.

Convalescent sera from chickens and ferrets and monoclonal antibodies have been used for determining antigenic relatedness of influenza viruses within the individual subtype (80). Such studies typically have used HI, enzyme-linked immunosorbent assays (ELISA), and/or virus neutralization tests. Monoclonal antibodies have been useful for the detailed study of individual antigenic epitopes such as comparing the HA of H1N1 viruses from turkeys and pigs to establish antigenic relatedness of their HAs (19,115).

Strain Nomenclature

Standard international nomenclature for the designation of influenza virus strains has been established (349). The naming of the influenza virus strains includes the type (A, B, or C), host of origin (except for human where the "host of origin" is omitted), geographic site, strain number (if any), and year of isolation followed by the antigenic subtype designating HA (H) and NA (N)

in parentheses (80). For example, a type A influenza virus isolated from chickens in Pennsylvania during 1983 and classified as H5N2 is designated "A/chicken/Pennsylvania/1370/83 (H5N2)."

Antigenic Variation of Strains—Drift and Shift

Human influenza viruses have a high frequency of antigenic variation in the surface glycoproteins (HA and NA) because of two phenomena, drift and shift. Such concepts were developed to explain the antigenic change that arose in influenza viruses within the human population over time (188). Some have proposed similar phenomena for AI viruses (115), but differences in the epidemiological nature of AI viruses and the lack of endemic infections in commercial poultry populations in developed countries makes complete extrapolation to all bird populations of doubtful scientific foundation.

Antigenic drift in influenza viruses arises from point mutations in the HA and/or NA genes that results in minor antigenic changes in the coding proteins (188). In mammals, immune pressure plays a role in selection of antigenic variants, but impact of immune pressure on antigenic change of AI viruses is less well documented. In vaccinated poultry populations, immune pressure may play a role in selecting antigenic variants (166), but for most commercial poultry in developed countries, infrequent exposure to AI viruses, short life spans, and the rare use of vaccines raise doubt about the importance of vaccine pressure for selecting variants. However, in areas where LPAI viruses are endemic, such as H9N2 LPAI viruses in the Middle East and Asia, infection by field viruses is widespread with the appearance of drift variants in the field. It is unclear whether such immunological pressure is from vaccine use or infections by circulating field viruses.

Antigenic shift arises from genetic reassortment between the gene segments of two influenza viruses that infect the same cell and results in the acquisition of new HA and/or NA antigens in a population with endemic influenza (188). The lack of endemic influenza in most commercial poultry raises doubt as to the importance of antigenic shift in generating new strains, but in LPM, antigenic shift in HA and/or NA subtypes has been documented. In addition, genetic reassortment has resulted in the exchange of viral genes other than those coding for the HA and NA, as reported for the Hong Kong H5N1 virus of chickens and geese (54,275,277). In wild ducks, mixed infections have been reported based on antigenic and molecular testing (119).

Immunogenicity or Protective Characteristics

The HA is the major antigen that elicits antibodies which protect against death and clinical signs. Such antibodies are HA subtype specific (i.e., neutralize influenza virus of homologous HA subtype in *in vitro* assays). *In vivo* protection is also HA subtype specific and can last for periods greater than 35 weeks (43). Antibodies produced against the NA provide protection against homologous NA subtypes in birds (177), but such protection is inferior to HA-induced protection.

Antibodies against the internal proteins, principally nucleoprotein, do not confer protection from death or clinical signs following challenge by HPAI viruses (340). However, some reports suggest immunization with NP can reduce titers of influenza virus replication in lungs during the late stages of the infectious process (149). This protection may be mediated by cytotoxic T lymphocytes. In addition, prior exposure of chickens to live H9N2 LPAI virus conferred some level of protection in chickens against H5N1 HPAI virus in Hong Kong markets through cell mediated immunity (240). However, such heterotypic immunity has not been of sufficient length and level for use in the field.

Genetic or Molecular

In the 1980s, individual AI virus strains and reassortant viruses were differentiated by examining migration patterns of RNA segments in polyacrylamide gel electrophoresis (80). In addition, mutations within gene segments of closely related viruses were identified by oligonucleotide mapping (25). However, in the 1990s, the technical ease of obtaining and analyzing genetic sequence data has resulted in an explosion of genomic information about AI viruses. Such information has included partial sequence data for most gene segments, frequent reporting of full-length sequence for some gene segments (HA, NA, M, and NS) and, in some situations, the complete nucleotide sequence of all eight gene segments (277). This has resulted in detailed phylogenetic comparisons of strains for purposes of molecular epidemiology, identification of reassortant gene segments, and identification of specific mutations and their correlation with biological properties. With development of high throughput sequencers in later 1990s and early 2000s, full length genome sequencing has become a common laboratory tool.

Pathotype

Based on pathogenicity (i.e., the ability to produce disease), AI viruses from poultry are classified into two pathotypes: 1) HP, and 2) LP (i.e., non-HP). This terminology was originally based on lethality in experimentally inoculated chickens, but in 1994, molecular and *in vitro* criteria were added to the definition (326). Previously, OIE-specified HPAI viruses on List A and LPAI viruses were not reported, but this system of categorization on Lists A and B has been eliminated and new AI categorization developed for international trade purposes. Because some H5 and H7 LPAI viruses have been shown to change to HPAI viruses during circulation in chicken or turkey populations, OIE has added H5 and H7 LPAI viruses to the International Animal Health Code (199). The OIE Terrestrial Code now lists "notifiable" AI (HPNAI and LPNAI) as follows (199):

1. HPNAI viruses have an IVPI in 6-week-old chickens greater than 1.2 or, as an alternative, cause at least 75% mortality in 4-to-8-week-old chickens infected intravenously. H5 and H7 viruses which do not have an IVPI of greater than 1.2 or cause less than 75% mortality in an intravenous lethality test should be sequenced to determine whether multiple basic amino acids are present at the cleavage site of the hemagglutinin molecule (HA0); if the amino acid motif is similar to that observed for other HPNAI isolates, the isolate being tested should be considered as HPNAI.

2. LPNAI are all influenza A viruses of H5 and H7 subtype that are not HPNAI viruses.

In addition, the OIE code creates by default a third category of AI viruses—non-H5 and non-H7 LPAI viruses—which are not reported to OIE, but may be reportable to national and state/provincial authorities. However, based on pathobiological criteria (e.g., disease, lesions and signalment), LPAI viruses are indistinguishable irrespective of the H and N subtype. Although the pathogenicity classification is specific for chickens, similar *in vivo* test results have been obtained for related birds in the order *Galliformes* (12,216). However, most AI viruses that are HP for chickens have been LP for domestic ducks except for some strains of the recent Asian H5N1 HPAI virus, which are also highly lethal for young domestic ducks, but not highly lethal in older ducks (12,129). Pathogenicity test results are specific for the host used in the test.

Prior to 1959, HPAI viruses ("fowl plague" viruses) were thought to be associated only with H7 viruses. Then H5 was found to cause the same disease in 1959 and 1961. Furthermore, the discovery of low virulence H5 AI viruses in turkeys during 1966–1968 (5,82,258) and H7 during 1971 (30) established that antigenic subtyping was not a predictor of high pathogenicity. Only a small percentage of H5 and H7 AI viruses have been HP. By contrast, all H1–4, H6, and H8–16 AI viruses have been of low virulence (i.e., LP) for birds. Although non-H5 and non-H7 subtypes have been LP by the OIE definition, in some cases they can cause severe and economically important disease in the field where secondary infection and other stressors impact disease development.

Laboratory Host Systems

The preferred method for isolation and propagation of AI viruses has been 9–11-days-old embryonating chicken eggs inoculated via the chorioallantoic sac (CAS) (302), but with some isolates, inoculation by the yolk sac route or inoculation onto the chorioallantoic membrane resulted in isolation where CAS route failed (352). In embryonating chicken eggs, AI viruses grow to high titers and have a cleaved HA (80). Most inactivated vaccines have been produced by cultivation in embryonating eggs.

Avian influenza viruses replicate in a limited number of cell culture systems (80). Primary cultures of chicken embryo fibroblasts (CEF) or kidney cells are most commonly used for plaque assays and virus neutralization tests. Madin-Darby canine kidney cell cultures have also been used. However, in CEF and some other cells, LPAI viruses require the addition of exogenous trypsin to the medium or agar overlay in order to cleave the HA and produce infectious virus (80). Absence of exogenous trypsin will produce plaques less than 1 mm in size or no plaques depending on the virus strain. The HPAI viruses do not require the addition of exogenous trypsin for cleavage of HA and the subsequent production of infectious virus.

The chicken (*Gallus gallus domesticus*) has been the most frequently used animal in laboratory studies to determine pathogenicity and study pathogenesis. Other commonly used laboratory species include the turkey (*Meleagridis gallopavo*), domestic duck (*Anas platyrhynchos*), house mouse (*Mus musculus*) and ferret (*Mustela putorius furo*). The mouse and ferret have been used as models to assess the risk of interspecies transmission of AI viruses to mammals (77), and the other species provide assessment of infections in natural hosts.

Pathogenicity

Clinical Groups in the Field

Although only two pathotypes of AI viruses can be demonstrated in the laboratory (HP and LP), natural infection by AI viruses results in a wide range of clinical outcomes which are dependent on virus strain, host species, host age, and environmental factors. From mortality patterns, clinical signs, and lesions in the field, AI can be categorized into four clinical groups: 1) highly virulent, 2) moderately virulent, 3) mildly virulent, and 4) avirulent. First, the highly virulent clinical group results from infection by HP H5 or H7 AI viruses usually in chickens or closely related gallinaceous birds and is expressed as a severe, highly fatal systemic disease that affects most organ systems including the nervous and cardiovascular systems. Morbidity and mortality approach 100%. Experimentally, the HPAI viruses reproduce the lesions and high mortality rates seen in the field (308). Second, the moderately virulent clinical group results from infection by LPAI viruses, of any HA or NA subtype, but also with co-infection by secondary pathogens (48,194). The mortality rates vary but range from 5–97% with the highest mortality occurring in young birds, reproductively active hens, or severely stressed birds (39,48,130). Lesions usually have been in the respiratory tract, reproductive organs, kidney, or pancreas (48,125,358). Some of these cases may have involved concurrent infection with bacteria that secreted proteases which cleaved HA of LPAI viruses, thus exacerbating the AI virus infection (252). Third, the mildly virulent clinical group results from infection by LPAI virus with low mortality and mild respiratory disease or drops in egg production. Mortality is usually less than 5%, and is typically in older birds. Fourth, the avirulent clinical group results from infections by LPAI viruses without any increased mortality or clinical signs. This has been most frequent with infections by LPAI viruses in wild birds of orders *Anseriformes* and *Charadriiformes* (283). In poultry, this has been seen following the introduction of a poorly host-adapted LPAI virus. Such an example would be the first cases of AI in range turkeys following exposure to wild waterfowl AI viruses that resulted in sero-conversion detected at slaughter without any previously noted clinical signs (283). Occasionally HP and LP viruses may appear together and this causes confusion in the field and the laboratory. In individual field situations, the clinical outcomes can be a mixture of the four clinical groups. For example, during the changing of a H5 or H7 LP to HPAI virus, gross lesions consistent with highly virulent AI will be seen in some dead birds, but the mortality rates will be low, similar to mildly virulent AI.

Effect of the Hemagglutinin Protein on Pathogenicity

The HA gene is the primary determinant of high pathogenicity in chickens, but a proper constellation of all eight gene segments is required for the maximal expression of virulence potential (36).

Table 6.6. Examples of genetic mechanisms for LP to HP change based on deduced amino acid sequence of hemagglutinin proteolytic cleavage sites in H5 and H7 AI viruses (modified from (236).

				Mechanism[1]					
Influenza virus	**Subtype**	**Pathotype**	**Amino acid sequence**	**1**	**2**	**3**	**4**	**5**	**References**
Typical H5 LPAI	H5	LP	PQ.....RETR*GLF						(236)
A/turkey/England/91	H5N1	HP	PQ....RKRKTR*GLF	X	X				(236)
A/chicken/PA/1370/83	H5N2	HP	PQ.....KKKR*GLF	X				X	(236)
A/tern/South Africa/61	H5N9	HP	PQRETRRQKR*GLF	X		X			(236)
A/chicken/Puebla/8623–607/94	H5N2	HP	PQ....RKRKTR*GLF	X	X				(101,126)
A/chicken/Queretaro/14588–19/95	H5N2	HP	PQRKRKRKTR*GLF	X	X				(101)
Typical H7 LPAI	H7	LP	PEIP.....KTR*GLF						(236)
A/chicken/Victoria/85	H7N7	HP	PEIP.....KKREKR*GLF			X			(236)
A/turkey/Italy/4580/99	H7N1	HP	PEIPKG....SRVRR*GLF			X			(48)
A/chicken/Chile/176822/02	H7N3	HP	PEKPKTCSPLSRCRETR*GLF[2]				X		(279)
A/chicken/Canada/AVFV2/04	H7N3	HP	PENPK ..QAYRKRMTR*GLF[3]				X		(207)

[1]Mechanisms: 1) substitutions of non-basic with basic amino acids; 2) insertions of multiple basic amino acids from codons duplicated from hemagglutinin cleavage site; 3) short inserts of basic and non-basic amino acids from unknown source; 4) non-homologous recombination with inserts which lengthen the proteolytic cleavage site; 5) loss of the shielding glycosylation site at residue 13.
[2]30 nucleotides from nucleoprotein of same virus gene coding 10 amino acid insert
[3]21 nucleotides from matrix of same virus gene coding 7 amino acid insert

In brief, the cleavage of the HA protein into the HA1 and HA2 proteins is essential for the virus to be infectious and produce multiple replication cycles. With LPAI viruses, only the trypsin-like proteases found in restricted anatomical sites, such as respiratory and intestinal epithelial cells or within secretions of the respiratory lumen, which are required to cleave the HA and thereby produce infectious virus, recognize the HA proteolytic cleavage site sequence. The HA proteolytic cleavage site of LPAI viruses has two non-consecutive basic amino acids at the carboxy-terminus of the HA1 and a glycosylation site at amino acid residue 13 that shields the proteolytic cleavage site. In contrast, H5 and H7 HPAI viruses have HA with a proteolytic cleavage site that is recognized and cleaved by ubiquitous furin proteases present in many cells of numerous visceral organs, the nervous system, and the cardiovascular system (268). Trypsin-like enzymes will also cleave the HA of HPAI viruses. These viruses have an altered HA proteolytic cleavage site structure at the carboxy-terminus of HA1 as compared to LPAI viruses (Table 6.6): 1) substitutions of non-basic with basic amino acids, 2) insertions of multiple basic amino acids from codons duplicated at the hemagglutinin cleavage site; 3) short inserts of basic and non-basic amino acids from unknown source; or 4) non-homologous recombination with inserts that lengthen the proteolytic cleavage site but which may or may not contain additional basic amino acids (Table 6.6) (48,101,126,211–213). In addition, loss of the shielding glycosylation site at residue Asn-11 may confer HP to some AI viruses if they have intermediate numbers of basic amino acids at the cleavage site (Table 6.6) (101,136).

In vitro tests have been used to predict pathogenicity potential and are based on structural variations in the proteolytic cleavage site of the hemagglutinin and its susceptibility to cleavage by different enzymes in various tissues. For example, the ability to produce large plaques in tissue culture, such as chicken embryo fibroblast cultures, without trypsin supplementation correlates with furin cleavage of HA and HP in chickens in *in vivo* tests, but LPAI viruses require the addition of exogenous trypsin to cleave the HA and produce large plaques (36). Detection of a cleaved HA in tissue cell cultures without trypsin by radioimmunoprecipitation correlates with HP (238). All HP and LPAI viruses produce cleaved HA in embryonating chicken eggs. Detection of multiple basic amino acids at the HA proteolytic cleavage site correlates with HP or potential to become HP (292,341)

An issue separate from HA cleavability is receptor binding between the receptor-binding site of the HA and the receptor on the host cells. This is a poorly understood phenomenon but impacts both host specificity (host-adaption) and cell or tissue tropism within the host. This may restrict virus replication to specific cells, tissues, and organs. Changes in the receptor-binding site of the HA have been shown to change the host range of an influenza virus (191). Both the virus and host impact receptor binding.

Mechanisms of Cellular Pathobiology

Based on morphologic and biochemical evidence, AI viruses exert pathological effect on avian cells by two mechanisms: necrosis or apoptosis (118,231,277). Necrosis has been identified in many cell types including kidney tubule cells, pancreatic acinar epithelium, cardiac myocytes, adrenal cortical cells, and pulmonary epithelial cells in chickens (277). Necrosis has been associated with intense virus replication and demonstration of abundant AI viral nucleoprotein in the nucleus and cytoplasm (303). Apoptotic cell death has been demonstrated in various cell culture systems and involved several cytokines including interferon-beta and transforming growth factor-beta (118,231,232, 311). *In vivo*, apoptotic cell death has been identified most often

in lymphocytes, especially in the absence of direct AI viral replication (277). However, apoptosis has been demonstrated in neurons, respiratory epithelium, and pulmonary alveolar cells of mice infected with mouse-adapted influenza viruses (185,186). In chicken embryos, apoptosis and necrosis may share similar biochemical features and indicate that differentiation morphologically and biochemically between them is not always easy nor clear (93).

Pathobiology and Epidemiology

Incidence and Distribution

Avian influenza viruses have a worldwide distribution with reports of isolations from Africa, Asia, Australia, Europe, and North and South America and serologic evidence of infection in penguins from Antarctic (80,184,264,308). Proceedings of the International Symposia on Avian Influenza have tabulated LP and HPAI outbreaks and incidences since 1981 (22,78,79,287, 307,309).

The most frequent reports of AI viruses has been free-flying aquatic birds, especially of the orders *Anseriformes* (ducks and geese) and *Charadriiformes* (shorebirds, gulls, terns, and auks), which are considered the biological and genetic reservoirs of all AI viruses (265). In these species, AI virus infections usually have caused no disease (LPAI viruses) with the exception of high mortality in common terns of South Africa during 1961 and infections and mortality in a variety of wild birds with H5N1 HPAI virus that originated from Asia in 1996 (Table 6.3). Dabbling ducks, especially mallards (*Anas platyrhynchos*), have the highest reported isolation rates of AI viruses with up to 60% of juvenile ducks being infected prior to migration in the late summer (122). This frequency decreases during migration with the lowest rate (0.4–31%) being seen in ducks on the wintering grounds (267). However, the frequency of infection in native, nonmigratory ducks increases when migratory ducks arrive in the wintering grounds (113,267). The H3, H4, H6, N2, N6, and N8 subtypes have been dominant among AI viruses isolated from free-flying ducks (122,151,225,255,265). For shorebirds (Order: *Charadriiformes*), the greatest number of isolations have been in the spring with a second peak during the fall migration (134). The dominant AI virus subtypes have been H3, H9, H11, H13, N2, N4, N8, and N9 (104,134,151). However, most combinations of the 16 HA and 9 NA subtypes have been reported in free-flying aquatic birds. AI viruses are infrequently isolated from wild terrestrial birds because they occupy non-aquatic ecosystems that do not favor maintenance of AI viruses (265).

AI viruses have been isolated sporadically from domestic poultry, most frequently chickens, turkeys, and ducks and captive wild birds held as caged pets, or in quarantine stations, private collections/reserves, and zoological parks (5,7). However, incidence and distribution vary greatly with geographic region, species, age of bird, time of year, and the environmental or agricultural system occupied.

Turkeys and other gallinaceous birds (including chickens) are not natural reservoirs of AI viruses (213,278). Humans have altered the natural ecosystems of birds through captivity, domestication, industrial agriculture, national and international commerce and nontraditional raising practices (283). This has created new niches for AI viruses and variations in the incidence and distribution of AI infections. Five distinct man-made ecosystems have been identified that have impacted AI virus ecology (283): 1) integrated indoor commercial poultry; 2) range-raised commercial poultry; 3) live poultry markets (LPM); 4) village, backyard and hobby flocks; and 5) bird collection and trading systems. The frequency of AI infections within each system has varied. For example, in most integrated commercial poultry systems in developed countries, AI has been a rare occurrence considering the 25–30 billion chickens raised each year (324). However, when AI infections do occur, they sometimes spread rapidly throughout the integrated system from farm to farm resulting in epidemics of HP (Table 6.3) or LPAI. In commercial poultry, outbreaks have been most frequently reported in turkeys, slightly less frequently in laying chickens, and rarely in other domesticated poultry. A vaccination and controlled marketing strategy has reportedly eliminated H5N2 HPAI virus from Mexico, but H5N2 LPAI virus continues to circulate in commercial chickens of Mexico (330). In some developing countries of Asia and the Middle East during the middle to late 1990s, H9N2 LPAI became endemic in commercial chickens. Since 2003, H5N1 HPAI has become endemic in many Asian countries, being maintained in village or rural poultry, especially domestic ducks.

In Minnesota, influenza outbreaks in turkeys have been associated with range rearing and the introduction of AI viruses from migratory waterfowl (112). However, the number of farms with infected turkeys has varied from year to year with a minimum of two infected flocks in 1983 and peak infections of 141 (1978), 258 (1988), and 178 (1995) flocks (110). In 1998 the industry decided to eliminate range rearing of turkeys and as a result, only 33 flocks were infected from 1996 to 2000 and most were infected from H1N1 swine influenza (109). However, migratory waterfowl exposure alone does not adequately explain year-to-year variations in LPAI outbreaks in turkeys. Individual virus strain and the host species (chicken versus turkey) impact interspecies transmission of AI viruses from migratory waterfowl. For example, the H7N2 LPAI virus in Virginia during 2002 produced a higher proportion of affected turkey then chicken farms and, in experimental laboratory tests, this virus was more contagious for turkeys than chickens as evident by requiring 100–250 times less virus to infect turkeys than chickens (319).

Prior to the advent of modern vertically integrated commercial poultry systems in the 1950s combined with common use of refrigeration for storage and shipping, most meat and egg type stock were raised locally in backyard and hobby flocks or small commercial farms with immediate slaughter and consumption (103). Such small local production and slaughter still exist today as the LPM system in developed countries, but total production is dwarfed by the vertically integrated commercial system. In both developed and developing countries, village and rural poultry and LPM systems have some of the highest AI virus infection rates (245). Historically, poorly controlled movement and lack of biosecurity caused AI to become endemic in some poultry populations, especially between 1900–1930 in Europe and some areas

of Asia (273). The 1924–1925 HPAI outbreak in the United States occurred in a LPM-type system but was eliminated by depopulation before becoming endemic (152). Endemic HPAI disappeared from in Europe by the mid-1930s (11). Recent surveys of poultry in LPM of Hong Kong, New York, and other large cities have indicated LPAI viruses have become endemic in these agricultural systems (239,244,245,318,334). LPMs were the site of a 1997 H5N1 AI outbreak in Hong Kong and a 1997 H5N2 HD outbreak in Italy, and the LPM was most likely the source of HPAI viruses that caused the 1983–1984 outbreak in the United States (51,245,277,334). Low pathogenicity H7N2 AI has become endemic in poultry of the Northeast USA LPM (1993–2006), but since the implementation of a control program, the rate of infection has declined. These LPMs were the source of H7N2 LPAI that crossed over to infect 24 commercial poultry farms in Pennsylvania between 1996–1998, 7 farms in Pennsylvania during 2001–2002, 197 farms in Virginia during 2002, a large layer company in Connecticut during 2003, a small layer farm in Rhode Island during 2003, and 3 broiler farms in Delmarva during 2004 (3,76,87,235,276,288,358).

Most influenza infections in domestic poultry have been from avian-origin influenza viruses. However, H1N1, H1N2 and H3N2 swine influenza viruses have infected turkeys, especially turkey breeders, causing significant disease problems (80,181, 281,312),

Natural and Experimental Hosts

Avian influenza viruses have been shown to naturally infect a wide variety of wild and domestic birds, especially free-living birds occupying aquatic habitats. Some AI infections have involved wild terrestrial birds, but these birds do not represent a major source or reservoir of AI viruses (265). In brief, AI viruses have been isolated from more than 90 species of free-living birds representing 13 different orders: *Anseriformes* (ducks, geese, and swans), *Charadriiformes* (e.g., shorebirds [turnstones and sandpipers], gulls, terns, puffins, and guillemots), *Ciconiiformes* (herons and ibis), *Columbiformes* (doves), *Falconiformes* (raptors), *Galliformes* (partridge and pheasant), *Gaviiformes* (loons), *Gruiformes* (coots and moorhen), *Passeriformes* (perching birds—e.g., mynahs, finches, and weaverbirds), *Pelecaniformes* (cormorant), *Piciformes* (woodpecker), *Podicipediformes* (grebe), and *Procellariiformes* (shearwater) (5,7,13,174,266). This represents 61% of known avian species, but the actual number of naturally infected species is most likely much greater (7). Most AI infections have not produced recognizable disease in free-living birds.

In man-made ecosystems (agriculture, caged, hobby flocks, and exhibition systems), infections have been reported in *Psittaciformes* (parrots, cockatoos, and parakeets), *Casuariiformes* (emu), *Struthioniformes* (ostrich), *Rheiformes* (rhea), and most domesticated *Galliformes* and *Anseriformes*. The latter two groups include chickens, turkeys, Japanese quail (*Coturnix japonica*), helmeted guineafowl (*Numida meleagris*), bobwhite quail (*Colinus virginianus*), pheasants (various species), chukar partridges (*Alectoris chukar*), geese (*Anser anser domesticus*) and ducks (mallards [*Anas platyrhynchos domesticus*] and Muscovy [*Cairina moschata domesticus*]) (80). Birds of the orders *Psittaciformes* probably are infected after capture and during mixing with infected birds at holding sites or in quarantine (80). Some infections of free-living *Passeriformes* (perching birds—starlings and sparrows) have been associated with outbreaks on poultry farms where they may have acquired infections from close contact with poultry (169,183).

Low pathogencity avian influenza viruses have caused epidemics of respiratory disease in mink, seals, and whales (45,88,102,116,158,172,337). Recently, the H5N1 HPAI virus has caused sporadic infections in tigers, leopards, house cats, Owston's palm civets, a stone martin and pigs (90). Most of these cases involved close contact or consumption of infected birds. A few cases of natural infections by AI viruses in humans have been reported (see "Public Health Significance").

In experimental studies, AI viruses have been shown to infect pigs, ferrets, rats, rabbits, guinea pigs, mice, cats, mink, nonhuman primates, and humans (33,80,82,120,142,246).

Transmission and Carriers

AI virus is excreted from the nares, mouth, conjunctiva, and cloaca of infected birds into the environment because of virus replication in the respiratory, intestinal, renal, and/or reproductive organs. In intranasally inoculated 3-to-4-week-old chickens, peak levels of HPAI virus recovery have been greatest from the oropharynx (swabs—$10^{4.2–7.7}$ EID_{50}/mL mean chicken embryo infective doses of respiratory secretions), and peak levels from the cloaca have been lower (swabs—$10^{2.5–4.5}$ EID_{50}/gm of feces) (291,297); LPAI viruses typically produce lower oropharynx (swabs—$10^{1.1–5.5}$ EID_{50}/mL) and cloacal (swabs—$10^{1.0–4.3}$ EID_{50}/mL) titers (291). With HPAI viruses, high virus levels in tissues of infected birds make consumption of carcasses through predation or cannibalism another source of virus transmission to susceptible birds. Titers in meat vary with virus strain, bird species and clinical stage of infection: 1) titers from dead chickens infected with 1983 H5N2 HPAI Pennsylvania virus had $10^{2.2–3.2}$ EID_{50}/gm of meat while 2003 H5N1 HPAI S. Korean virus had $10^{5.5–8.0}$ EID_{50}/gm of meat, and 2) H5N1 HPAI viruses produced different titers in clinically normal ($10^{2.0–3.4}$ EID_{50}/gm) or sick ($10^{4.0–6.0}$ EID_{50}/gm) domestic ducks (291,315).

The virus is transmitted by direct contact between infected and susceptible birds or indirect contact through aerosol droplets or exposure to virus-contaminated fomites (80). Aerosol generation from the respiratory tract is a significant mode of transmission because of high virus concentrations in the respiratory tract, but the large volume of lower concentration AI virus in infected feces makes fomites a major mode of transport. Thus, AI viruses are readily transported to other premises by people (contaminated shoes and clothing) and equipment shared in production, live-haul, or live-bird marketing (80).

Influenza viruses exhibit varying degrees of adaptation to individual host species with frequent and easy intraspecies transmission (283). However, interspecies transmission does occur, especially between closely related host species in the same taxonomic family, such as chickens, turkeys, guinea fowl, and quail of the order *Galliformes*, family *Phasianidae*. Interspecies transmission can occur across different orders within the same class

such as with free-flying duck-(Order: *Anseriformes*)-to-turkey (Order: *Galliformes*), but this is less frequent than occurs with closely related host species (283). Furthermore, interspecies transmission between different phylogenetic classes is even less frequent as has occurred rarely with chicken-to-human (283). One exception to the preceding rule has been the ease and frequency of transfer of swine H1N1 and H3N2 viruses to turkeys when the two species were raised in close geographic proximity (181,280,283,312). Obviously, many factors such as geographic restriction of host distribution, intermixing of species, age and density of birds, weather, and temperature also impacted the ability of the AI virus to move within and between host species and affected the overall incidence of infections (283).

Sources of infection for the initial introduction of the influenza virus into commercial poultry flocks (i.e., primary infections) include: 1) other domestic and confined poultry, 2) migratory waterfowl and other wild birds, 3) domestic pigs, and 4) companion or pet birds (5,7). The relative risk associated with each of these sources varies depending on the likelihood of direct or indirect contact with commercial poultry. First, the LPM system poses a significant risk to the introduction of LP and HPAI viruses into the commercial integrated poultry systems. Theoretically, transmission could occur by airborne dissemination as proposed in some AI outbreaks (69). However, high volume air sampling during the 1983–1984 H5N2 HPAI outbreak in the United States failed to yield influenza virus when samples were taken more than 45 meters downwind from an infected house (41). This suggests airborne transmission may have a limited role in most interflock dissemination of AI virus as compared to mechanical movement of fomites on equipment, clothing, or shoes (41). Especially high risk is movement of dead infected birds from farms through a shared rendering system or from the farm for burial without adequate sealing and decontamination of transport vehicles (37). Second, introduction of AI viruses (especially LPAI viruses) from wild birds, especially waterfowl, has been documented (112). The source is suspected to be contaminated feces from the ducks either through direct contact with poultry or indirectly through contamination of feed or water (121). The transmission potential of AI viruses from wild waterfowl emphasizes the need for producers of domestic, commercial poultry to provide separation between domestic and wild bird populations (80). Third, turkeys can be infected by introduction of H1N1, H1N2 or H3N2 and, potentially other subtypes of swine-origin influenza viruses, either by mechanical methods or potentially via humans infected with swine-origin influenza viruses (80). Fourth, AI viruses have been recovered from caged birds, usually during quarantine, but transmission for this source to poultry has not been documented as has occurred with Newcastle disease virus (80). To minimize the risk of introduction and dissemination of AI viruses, producers should raise only one species of bird in an individual operation, have an all-in-all-out production system, or add new birds only after testing and quarantine and practice high degree of biosecurity.

Secondary dissemination of AI viruses during an outbreak can be by mechanical transmission of virus on fomites, movement of infected poultry, or in some situations possibly airborne dissemination,. Wild birds may play a major role in initial introduction of AI viruses in domestic poultry, but once established or adapted in commercial or LPM poultry, wild birds have had a very limited or no role in secondary dissemination (117,193). However, with the recent H5N1 HPAI viruses, wild birds have been infected and could serve a role in flock to flock spread in village or rural poultry systems.

Although horizontal transmission of AI viruses commonly occurs, proof of vertical transmission is lacking (80). However, HPAI virus infection of hens has resulted in virus recovery from the eggshell surface and the internal contents of the eggs (46). In experimental studies with HP H5N2 AI virus from Pennsylvania, most eggs laid on days 3 and 4 post-inoculation contained virus (28). However, AI viruses are embryo lethal, and hatching of internally contaminated eggs has never been demonstrated. Cleaning of fecal material and disinfection of eggshells may be necessary to prevent hatchery-associated dissemination of AI viruses. Most LPAI and HPAI viruses cause reduction or cessation, respectively, of egg production further limiting the potential for vertical transmission of AI virus.

Successful experimental routes of exposure include aerosol, intranasal, intrasinus, intratracheal, oral, conjunctival, intramuscular, intraperitoneal, intracaudal air sac, intravenous, cloacal, and intracranial administration of the various viruses (80).

In experimental studies, AI virus has been shown to replicate and be excreted from individual chickens for up to 36 days (304), and turkeys for up to 22 days (83,124). However, on a population basis, AI virus can be maintained for much longer time periods in agricultural operations or can re-emerge after a significantly stressful event. For example, a LP H7N2 AI virus in Pennsylvania during 1997–1998 was recovered from dead chickens collected from a layer flock with normal mortality six months after the initial AI infection on the farm and in another flock eight weeks after the induction of a molt (358). Once a flock is infected, it should be considered a potential source of virus for life. In wild waterfowl, AI viruses are maintained by passage in susceptible birds throughout the year, with peak prevalence in migratory waterfowl being in juvenile birds prior to fall migration (80). Prevalence of AI in migratory waterfowl AI is low as they arrive in the wintering grounds, but upon arrival, they infect susceptible resident waterfowl which go through their own cycle of infection (113,267). Thus, resident ducks contribute to the generation of virus during the winter and this source might reinfect migrating waterfowl prior to the spring migration.

Incubation Period

The incubation periods for the various diseases caused by these viruses range from as short as a few hours in intravenously inoculated birds to 3 days in naturally-infected individual birds and up to 14 days in a flock (80). In timed studies, intranasally inoculated chickens with H5N1 HPAI virus from Mongolia producted clinical signs within 24 hrs. The incubation period is dependent on the dose of virus, the route of exposure, the species exposed, and the ability to detect clinical signs (80). However, for international regulatory purposes, OIE recognizes 21 days as the

incubation period (199) where incubation period is defined as: the time from exposure to the onset of clinical signs, however this criteria may not be applicable to all AI viruses, especially LPAI viruses. Many infections by LPAI viruses do not cause clinical disease in all ages and all species of birds. "Infectious period", as defined as the time from exposure or detection of the virus to when the virus is no longer detected, may be more applicable for control and eradication purposes, especially in dealing with LPNAI viruses.

Clinical Signs

The pathotype of AI virus (LP or HP) has a major impact on the clinical manifestation of the disease. However, clinical signs of disease are extremely variable and depend on other factors including host species, age, sex, concurrent infections, acquired immunity, and environmental factors (80).

Low Pathogenicity Avian Influenza Viruses

Most infections by LPAI viruses in wild birds produce no clinical signs. However, in experimental studies in mallard ducks, LPAI virus infections suppressed T-cell function and produced a one week depression in egg production (163,164).

In domestic poultry (chickens and turkeys), clinical signs reflect abnormalities in the respiratory, digestive, urinary, and reproductive organs. The most frequent signs represent infection of the respiratory tract and include mild to severe respiratory signs such as coughing, sneezing, rales, rattles, and excessive lacrimation. In layers and breeders, hens may exhibit increased broodiness and decreased egg production. In addition, domestic poultry will exhibit generalized clinical signs including huddling, ruffled feathers, depression, decreased activity, lethargy, decreased feed and water consumption, and occasionally diarrhea. Emaciation has been reported but is infrequent because AI is an acute, not a chronic disease.

In ratites, LPAI viruses produced similar respiratory signs as with poultry and in some cases green diarrhea or "urine" (17,52, 131,173,202,205,295).

High Pathogenicity Avian Influenza Viruses

In wild birds and domestic ducks, most HPAI viruses either do not replicate or replicate to a limited degree and produce few clinical signs because of poor adaptation to non-gallinaceous species. There are two major exceptions to this rule: 1) the 1961 H5N3 HPAI outbreak in common terns in South Africa, which produced sudden death without any other clinical signs (35), and 2) some of the recent H5N1 HPAI viruses in wild birds and non-gallinaceous poultry produced mostly neurological signs, depression, anorexia and sudden death (167,320). Occasional sporadic, isolated cases of mortality have been reported in wild birds with HPAI viruses.

In domestic chickens, turkeys, and related galliformes, clinical signs reflect virus replication and damage to multiple visceral organs and cardiovascular and nervous systems. However, clinical manifestations vary depending on the extent of damage to specific organs and tissues (i.e., not all clinical signs are present in every bird). In most cases in chickens and turkeys, the disease is fulminating with some birds being found dead prior to observation of any clinical signs. If the disease is less fulminating and birds survive for 3–7 days, individual birds may exhibit nervous disorders such as tremors of head and neck, inability to stand, torticollis, opisthotonus, and other unusual positions of head and appendages. The poultry houses may be unusually quiet because of decreased activity and reduction in normal vocalizations of the birds. Depression is common as are significant declines in feed and water consumption. Precipitous drops in egg production occur in breeders and layers with typical declines including total cessation of egg production within six days. Respiratory signs are less prominent than with LPAI viruses but can include rales, sneezing, and coughing. Other galliforme birds have similar clinical signs but may live longer and have evidence of neurologic disorders such as paresis, paralysis, vestibular degradation (torticollis and nystagmus), and general behavior aberrations (216).

In ostriches (*Struthio camelus*), reduced activity and appetite, depression, ruffled feathers, sneezing, hemorrhagic diarrhea and open mouth breathing have been reported (47,52,63,64,173). In addition, some birds were uncoordinated, exhibited torticollis, and had paralysis of the wings and tremors of the head and neck.

Morbidity and Mortality

In chickens, turkeys, and related gallinaceous birds, morbidity and mortality rates are as variable as the signs and are dependent on virus pathogenicity and the host as well as age, environmental conditions, and concurrent infections (80). For the LPAI viruses, high morbidity and low mortality rates are typical. Mortality rates are usually less than 5% unless accompanied by secondary pathogens or if the disease is in young birds. For example, in the 1999 Italian H7N1 LPAI outbreak, mortality rates as high as 97% were observed in turkey poults less than 4 weeks of age when accompanied by secondary pathogens (48).

With the HPAI viruses, morbidity and mortality rates are very high (50–89%) and can reach 100% in some flocks. Typically, the virus spreads rapidly among poultry housed on the floor with peak mortality (70–100%) occurring in 3–5 days of first clinical signs, but in poultry housed in cages, the virus spreads slower through the house with peak mortality taking 10–15 days. With the H5N1 HPAI viruses, the mean death times in experimental studies (intranasal inoculation) are much shorter for chickens and turkeys than for other gallinaceous birds (219). In domestic ducks, the mortality from H5N1 HPAI viruses is dependent on virus strain and the age of the ducks. The H5N1 HPAI viruses from 1997–2001 did not cause illness or death in intranasally-inoculated ducks but many viruses from 2001–2006 caused varying mortality rates in 2–3 week old ducklings (60,60,167,274, 320). With specific viruses from Hong Kong in 2002, intranasal inoculation caused mortality, but in 5–6-week-old ducklings, either no deaths or low mortality was observed (299). This experimental variation based on age explains why mortality rates in domestic ducks and geese in the field have been low (247,355).

In ostriches, LP and HPAI viruses usually produce moderate morbidity and low mortality rates (47). Typically, the morbidity and mortality have been highest in young birds (<3 months) with mortality of 30% being seen (47), but mortality rates as high as

80% have been reported for LPAI viruses in chicks less than one month of age (17).

In wild birds, particularly waterfowl, neither LPAI or HPAI viruses usually produce mortality or morbidity. Occasionally, dead wild birds (passerines) have been identified on farms with HPAI outbreaks. High mortality was reported in the outbreak in South African terns during 1961. Mortality has been reported in a variety of wild birds associated with the recent H5N1 HPAI viruses in Asia, Africa and Europe. In some instances, the mortality has been sporadic involving individual birds such as pigeons (*Columbia livia*), or with some species such as swans and geese, large numbers of birds have died (61,86,247,336). Experimental studies have demonstrated great variation in the production of illness and death in wild birds, especially ducks (38,219). However, the exposure, morbidity and mortality rates are unknown since adequate surveys have not been conducted to determine the impact of H5N1 HPAI viruses on wild bird populations.

Pathology

Numerous reviews have been published on the pathology of AI virus (5,7,125,182,219,272,273). Details of field outbreaks and experimental studies have been published and are summarized in this section (2,22,31,47,48,50,52,66,80,82,132,145,146,152, 162–164,180,202,205,213,216–218,220,242,256,257,277, 282,289,295,296,301,303–306,308,320). The lesions in wild birds have been reported for some H5N1 HPAI viruses, but such information is beyond the scope of this chapter.

Gross

Gross lesions have been extremely variable with regard to their location and severity, depending greatly on the host species, pathogenicity of the infecting virus, and presence of secondary pathogens. Most frequently, descriptions of gross lesions have been provided for naturally occurring or experimental infections in chickens and turkeys. Until the late 1990s, few descriptions were available for other host species such as quail, ducks, geese and ratites.

Low Pathogenicity Avian Influenza Viruses. In gallinaceous poultry, the most frequent lesions are in the respiratory tract, especially sinuses, and are characterized as catarrhal, fibrinous, serofibrinous, mucopurulent, or fibrinopurulent inflammation. The tracheal mucosa can be edematous with congestion and occasionally hemorrhages. Tracheal exudates may vary from serous to caseous, with occasional occlusion of airways and resulting asphyxiation. Fibrinous to fibrinopurulent air sacculitis may be present. The fibrinopurulent inflammation usually is accompanied by secondary bacterial infections. The infraorbital sinuses may be swollen with mucous-to-mucopurulent nasal discharge. Fibrinopurulent bronchopneumonia can result when accompanied by secondary pathogens such as *Pasteurella multocida* or *Escherichia coli*.

Catarrhal to fibrinous inflammation may be noted in the air sacs and coelomic cavity ("peritoneal cavity"), and "egg yolk peritonitis" may be observed. Catarrhal to fibrinous enteritis may be observed in the ceca and/or intestine, especially in turkeys. Inflammatory exudates may be found in the oviducts of laying birds, and the last few eggs laid will have reductions in calcium deposition within the eggshells. Resulting eggs may be misshapen and fragile with loss of pigmentation. Ovaries will undergo regression, beginning with hemorrhage in the large follicles and progressing to colliquation. The oviduct may be edematous and contain catarrhal to fibrinous luminal exudates before undergoing involution. In a few cases in laying hens and intravenous inoculated chickens, swollen kidneys occurred and were accompanied by visceral urate deposition ("visceral gout").

Sporadically, other lesions have been reported including firm pancreas with pale mottling and hemorrhage, usually in turkeys.

In domestic ducks and geese, LPAI viruses may produce lesions in the respiratory tract such as sinusitis, conjunctivitis, and other respiratory lesions. Co-infections with bacteria are common.

In rheas (*Rhea americana*) and emus (*Dromaius novaehollandiae*), LPAI virus infection produced ocular discharge; fibrinous sinusitis, tracheitis, and air sacculitis; interstitial pneumonia; congested visceral organs; hemorrhage in trachea; and occasional fibrinous perihepatitis and pericarditis.

Highly Pathogenic Avian Influenza Viruses. In gallinaceous poultry, HPAI produce a variety of edematous, hemorrhagic and necrotic lesions in visceral organs and the skin (see Fig. 6.2). Although, when death is peracute, no gross lesions may be observed. In chickens, swelling of the head, face, upper neck, and feet may be observed which results from subcutaneous edema and may be accompanied by petechial-to-ecchymotic hemorrhages. Periorbital edema may be seen. Necrotic foci, hemorrhage, and cyanosis of the non-feathered skin have been reported, especially wattles and combs. Lesions in visceral organs vary with virus strain but most consistently are represented by hemorrhages on serosal or mucosal surfaces and foci of necrosis within parenchyma of visceral organs. Especially prominent are hemorrhages on the epicardium, in pectoral muscles, and in mucosa of the proventriculus and ventriculus. With the H5N1 HPAI viruses, necrosis and hemorrhage in Peyer's patches of the small intestine were common as was reported with outbreaks of fowl plague in the early 1900s. In addition, these strains of HPAI viruses tend to produce more severe hemorrhage and edema in the lungs.

With most HPAI viruses, necrotic foci are common in pancreas, spleen, and heart, and occasionally in liver and kidney. The kidney lesions may be accompanied by urate deposits. Lungs have focal ventral to diffuse interstitial pneumonia with edema. The lungs can be congested or hemorrhagic. The cloacal bursa and thymus are usually atrophic.

In ostriches, HPAI viruses produced edema of head and neck, severe hemorrhagic enteritis, enlarged and firm pancreas, mild to severe air sacculitis, hepatitis, peritonitis, renomegaly, and splenomegaly.

Microscopic

Low Pathogenicity Avian Influenza Viruses. In poultry, LPAI viruses produce pneumonia varying in character from ventromedial, fibrinocellular-to-peribronchiolar lymphocytic. In severe cases, the pneumonia may be diffuse with air capillary edema.

Heterophilic to lymphocytic tracheitis and bronchitis have been common. On intravenous (IV) or intranasal inoculation and in field cases in chickens, nephrosis and nephritis have been reported. However, this renal tropism is virus-strain specific and most consistently produced with IV inoculation. In turkeys, experimental and natural cases of pancreatitis with acinar necrosis have been seen, especially with the 1999 Italian H7N1 AI virus. Pancreatitis is less common in chickens than turkeys. Birds that die from LPAI have lymphocyte depletion and necrosis or apoptosis of lymphocytes in the cloacal bursa, thymus, and spleen, whereas other tissues such as trachea and nasal cavity have lymphocyte accumulations. Viral antigen is rarely seen in lymphocytes but is commonly demonstrated in necrotic respiratory epithelium, renal tubule epithelium, and pancreatic acinar epithelium. The latter is primarily in IN inoculated chickens.

In rheas (*Rhea americana*), the LPAI viruses produce heterophilic to pyogranulomatous sinusitis, bronchitis, and pneumonia with necrosis of respiratory epithelium. In ostriches, lesions of splenic and hepatic necrosis, enteritis, and sinusitis were seen.

Highly Pathogenic Avian Influenza Viruses. Lesions in natural outbreaks have been reported and have been reproduced in experimental studies with chickens. Histologic lesions are most consistent in tissues having gross lesions. Specific histopathologic descriptions for experimental studies vary with individual viruses as a result of variations in inoculum doses, strain of chicken, route of inoculation, and passage history. Basically, the histologic lesions consist of multi-organ necrosis and/or inflammation. The most consistent and most severely affected tissues are brain, heart, lung, pancreas, and primary and secondary lymphoid organs. Lymphocytic meningoencephalitis with focal gliosis, neuronal necrosis, and neuronophagia are common, but edema and hemorrhage may be seen. Focal degeneration to multifocal-diffuse coagulative necrosis of cardiac myocytes has been reported, usually with accompanying lymphohistiocytic inflammation. Lesions in the brain and heart have abundant associated influenza virus proteins in neurons and myocytes, respectively. Other common lesions associated with AI virus replication include necrosis in skeletal myofibers, kidney tubules, vascular endothelial cells, corticotrophic cells of adrenal, and pancreatic acinar cells. If the birds survive for 3–5 days, the quantity of necrosis is reduced and the intensity of lymphohistiocytic inflammation is increased. In lymphoid tissue, necrosis, apoptosis, and depletion are common in cloacal bursa, thymus, and spleen, but AI viral antigen is rarely seen in lymphocytes. The lesions in respiratory tract vary widely from minimal to severe. The non-feathered skin contains numerous microthrombi within dermal and hypodermal capillaries and small blood vessels. This is accompanied by vasculitis, perivascular to generalized edema, subcutaneous edema, and necrosis of capillary endothelium. The epidermis has various stages of vesicle formation progressing to full-thickness necrosis.

In gallinaceous species other than chickens and turkeys, lesions are similar to above, but in general, since the birds survive longer than chickens or turkeys, the necrosis and inflammation are more common and prominent in tissues.

In ostriches, HPAI viruses produced coagulative necrosis in spleen, kidney, and liver. Fibrinoid necrosis was common in the arterioles of the brain and spleen. The pancreas had necrosis of acinar cells with mild mononuclear cell inflammation and fibrosis. Foci of malacia and neuronophagia were present in brains, and necrotic and hemorrhagic lesions were in the intestine.

Pathogenesis of the Infectious Process

First, in poultry, the process begins by inhalation or ingestion of infectious LP or HPAI virions. Because trypsin-like enzymes in respiratory and intestinal epithelial cells allow cleavage of the surface hemagglutinin, multiple replication cycles occur in respiratory and/or intestinal tracts with release of infectious virions. In gallinaceous poultry, the nasal cavity is a major site of initial replication.

Second, with HPAI viruses, after initial replication in respiratory epithelium, the virions invade the submucosa, entering capillaries. The virus replicates within endothelial cells and spreads via the vascular or lymphatic systems to infect and replicate in a variety of cell types in visceral organs, brain, and skin. Alternatively, the virus may become systemic before having extensive replication in vascular endothelial cells. The virus is present in the plasma, red and white blood cell fractions. Macrophages appear to play a role in systemic virus spread. The presence of a hemagglutinin proteolytic cleavage site that can be cut by ubiquitous furin-like cellular enzymes is responsible for this pantropic replication. Clinical signs and death are due to multiple organ failure. Damage caused by AI viruses is the result of one of four processes: 1) direct virus replication in cells, tissues, and organs; 2) indirect effects from production of cellular mediators such as cytokines; 3) ischemia from vascular thrombosis, and 4) cardiovascular collapse from coagulopathy or disseminated intravascular coagulation.

Third, for the LPAI viruses, replication usually is limited to the respiratory or intestinal tracts. Illness or death is most often from respiratory damage, especially if accompanied by secondary bacterial infections. Sporadically in some species, the LPAI viruses spread systemically, replicating and causing damage in kidney tubules, pancreatic acinar epithelium, oviduct and other organs with epithelial cells having trypsin-like enzymes.

Pathogenesis of the infection process is less well understood in non-gallinaceous birds.

Immunity

Active

Infection with AI viruses as well as immunization with vaccines elicits a humoral antibody response at both the systemic and mucosal levels (278). This includes a systemic IgM response by 5 days post-infection, followed shortly by an IgG response. The mucosal immune response is poorly characterized (278). The intensity of the antibody response varies with bird species (i.e., chickens > pheasant > turkeys > quail > ducks) (114,278). However, reports of serological response in ducks varies from poor antibody response and no hemagglutination inhibition (HI) antibodies (140,316) to 29.5% HI positive ducks on a farm near the 1992 H7N3 outbreak in Australia (344).

Antibodies against the surface proteins (HA and NA) are neu-

tralizing and protective (278). Protection has been primarily associated with antibodies directed against the HA protein. However, antibodies directed against either HA, NA, or both prevent clinical signs and death following challenge with HPAI viruses with homologous HA or NA subtypes. The level of protection against mucosal infection and subsequent shedding of challenge virus may depend on the degree of antigenic (or proteins sequence) similarity between HA of vaccine and challenge virus (293,297,300). Duration of protection is unknown, but in layers, protection against clinical signs and death has been demonstrated to at least 30 weeks following a single immunization (43). Birds that have recovered from field exposure are protected from the same HA and NA subtypes. Some birds, such as waterfowl, turkeys and long-lived chickens (layers and breeders) may require multiple vaccinations to maintain adequate protection.

Immune response against viral internal proteins has not been shown to prevent clinical signs or death but may shorten the period of virus replication and shedding (149). However, the mechanism of this limited protection is unknown but may be the result of cell-mediated immunity. A recent experimental study with inactivated H9N2 AI virus demonstrated short-term protection in chickens against HP H5N1 AI challenge virus, but immunization did not totally block virus replication in the digestive tract (240). Cell-mediated immunity was responsible for the protection.

Passive

Studies on protection by maternal antibodies to homologous HA or NA have not been reported, but based on evidence available for other avian pathogens, protection against clinical signs and death from homologous AI viral challenge is probable for the first two weeks after hatching.

Diagnosis

A definitive diagnosis of AI is established by 1) direct detection of AI viral proteins or genes in specimens such as tissues, swabs, cell cultures, or embryonating eggs; or 2) isolation and identification of AI virus. A presumptive diagnosis can be made by detecting antibodies to AI virus. During outbreaks of HPAI, mortality rates, clinical signs and lesions may be useful as part of the case definition in deciding which farms to quarantine and possibly for depopulation of birds for eradication purposes.

Sample Selection and Storage

Avian influenza viruses are commonly recovered from tracheal, oropharyngeal or cloacal swabs of either live or dead birds, because most HP and LPAI viruses replicate in the respiratory and intestinal tracts. The swabs should be placed in a sterile transport medium containing high levels of antibiotics to reduce bacterial growth (302). Tissues, secretions, or excretions from these tracts are appropriate for virus isolation or detection. Tissues can be collected and placed into sterile plastic tubes or bags. In the examination of organs for virus, efforts should be made to collect and store internal organs separately from the respiratory and intestinal tract tissues because isolation of virus from internal organs may be an indication of systematic spread and is most often associated with the HPAI viruses. In the case of systemic infections produced by HPAI viruses, virtually every organ can yield virus because of the high levels of viremia or replication in parenchymal cells.

If the samples for virus detection can be tested within 48 hours after collection, they may be kept at 4°C; however, if the samples must be held for additional time, storage at –70°C is recommended. Before testing for virus, tissues should be ground as a 5–10% suspension in the transport medium and clarified by low-speed centrifugation.

Direct Detection of AI Viral Proteins or Nucleic Acids

The direct demonstration of influenza virus RNA or viral proteins in samples from animals is routinely used as a diagnostic screening test. Several commercial and laboratory specific antigen detection kits are available for detection of influenza A nucleoprotein and have been used to detect influenza viral antigen in avian specimens and allantoic fluid of inoculated embryonating chicken eggs (70,148,251,351). These antigen capture immunoassays vary in sensitivity with the best tests being 3–4 log less sensitive than virus isolation (351). Polyclonal and monoclonal antibodies are useful for localizing viral antigen in tissues by immunofluorescent or immunoperoxidase staining methods (250,256,328), and radiolabeled gene probes for *in situ* hybridization can locate cells involved in viral replication in tissues of infected birds (327). Reverse transcriptase polymerase chain reaction (RT-PCR) and real-time RT-PCR (RRT-PCR) methods have been developed and used in some laboratories for experimental studies and field case diagnosis of AI (3,262,354). The RRT-PCR has a 3 hour test time and a sensitivity and specificity comparable to virus isolation procedures (97). This technology has accelerated influenza diagnosis and field monitoring. In the USA, screening of tracheal or oropharyngeal samples is done using a matrix gene RRT-PCR test and if positive, the samples are tested with H5 and H7 subtype specific RRT-PCR tests (263)

Virus Isolation

Methods for the isolation and identification of influenza viruses have been described in detail (55,80,302). Chicken embryos, 9–11 days old, are inoculated via the allantoic cavity with approximately 0.2 mL of sample. In some cases, yolk sac inoculation has yielded viruses when allantoic cavity inoculation has failed (352).

The death of inoculated embryos within 24 hours after inoculation usually results from bacterial contamination or inoculation injury, and these eggs should be discarded. A few viruses may grow rapidly and kill the embryos by 48 hours; however, in most cases the embryos will not die before this time. After 72 hours, or at death, the eggs should be removed from the incubator, chilled, and allantoic fluids collected. The presence of virus is demonstrated by hemagglutinating activity using chicken erythrocytes, however the presence of NDV must be excluded.

Generally, if virus is present in a sample, there will be sufficient growth in the first passage to result in hemagglutination, and repeated passage is unnecessary. Repeated passage of samples increases the risk of cross-contamination in the laboratory.

Long-term storage of viruses should be done at –70°C or below. Lyophilization of viruses is also appropriate for long-term storage; however, these stocks should be tested periodically to ensure infectivity.

Virus Identification

Standardized methods for testing the egg fluids for the presence of hemagglutinating activity using chicken erythrocytes by macro- or micro-techniques are employed (58,80,302). Allantoic fluid positive for hemagglutination is used for virus identification.

It is important to determine whether the hemagglutinating activity detected in the allantoic fluid is due to influenza virus or other hemagglutinating viruses, such as paramyxoviruses like Newcastle disease virus (NDV). Thus, the isolate is tested in HI assays against Newcastle disease and other antiseram. If negative, the virus then is tested for the presence of the type A specific antigen to establish that an influenza A virus is present. The type-specific NP (nucleoprotein) or matrix protein may be detected by the double immunodiffusion test (26,74), the single-radial-hemolysis test (74) or commercial antigen capture immunoassay. Monoclonal antibodies that react with the nucleoprotein or matrix proteins have proven useful in identifying these antigens in ELISA (332).

The next step in the identification procedure is to determine the antigenic subtype of the surface antigens, HA and NA. The NA subtype is identified by a micro-NI assay with antisera prepared against the nine known NAs (204,302,329).

The HA is identified in the HI test (302) using a panel of polyclonal antisera prepared against whole virus representing the 16 distinct HA subtypes. Subtyping is facilitated by using antisera against the HA alone (i.e not the whole virus) or against reassortant viruses with heterologous NAs; this helps avoid steric inhibition due to antibodies against the NA (137,139). An influenza virus with a new HA would not be detected in tests using antisera to the known HA subtypes. Therefore, it is essential to confirm that the unknown hemagglutinating agent is an influenza virus using the type-specific test described previously.

Final identification is most commonly accomplished by state, federal, or OIE influenza reference laboratories.

Serology

Serologic tests are used to demonstrate the presence of AI-specific antibodies, which may be detected as early as seven days after infection. Several techniques are used for serologic surveillance and diagnosis. In serologic surveillance programs, a double immunodiffusion test (agar gel immunodiffusion or AGID) for the detection of anti-NP antibody is frequently used, because this detects antibodies to type A-specific antigens shared by all influenza A viruses. ELISA assays have been developed to detect antibodies to AI viruses (1,34,92,179,241,259,357). ELISAs are commercially available for detecting antibody to influenza from chickens and turkeys. Once influenza is detected by immunodiffusion or ELISA, HI tests can be used to determine the HA subtype.

In serologic assays, be aware that there is considerable variation in the immune response among the various avian species. For example, antibodies to the NP are generally prominent in turkeys and pheasants but may be undetectable in ducks known to have been infected (253). In addition, antibodies may be induced in ducks, as well as other species, which fail to be detected in conventional HI tests performed with intact virus (141,171).

The sera of many species contain nonspecific inhibitors that may interfere with the specificity of the HI and other tests. Because these inhibitors are especially active against certain viruses, they present a very practical problem in serologic testing and the identification of viruses. Therefore, sera should be treated to reduce or destroy such activity, although it should be recognized that some treatments may lower specific antibody levels. The two most commonly used treatments for these inhibitors have been receptor destroying enzyme (RDE) and potassium periodate (58,74). In addition to the non-specific inhibitors of hemagglutination, sera from other bird species, such as turkey and goose, may cause non-specific agglutination of the chicken erythrocytes used in the HI test. This may mask low levels of HI activity. Such hemagglutinating activity can be removed by pretreatment of the serum with chicken erythrocytes (192). This problem may sometimes be avoided by using erythrocytes in the HI test of the same species as the serum being tested.

Differential Diagnosis

Because of the broad spectrum of signs and lesions reported with infections by AI viruses in several species, a definitive diagnosis must be made by virologic and serologic methods. For HPAI viruses, other causes of high mortality must be excluded such as Newcastle disease, septicemic fowl cholera, heat exhaustion, water deprivation and some toxins. For LPAI viruses, other causes of respiratory disease and drops in egg production must be investigated such as lentogenic Newcastle disease virus, avian pneumovirus and other paramyxoviruses, infectious laryngotracheitis, infectious bronchitis, chlamydia, mycoplasma, and various bacteria. Concurrent infections with other viruses or other bacteria have been commonly observed (80).

Intervention Strategies

Management Procedures

There are three different goals or outcomes in the control of AI: 1) prevention, 2) management, and 3) eradication (284). These outcomes are accomplished based on strategies using combinations of five specific components: 1) education, 2) biosecurity, 3) diagnostics and surveillance, 4) elimination of infected poultry, and 5) decreasing host susceptibility. How effective each strategy is at controlling AI is dependent upon how many of the five components are used and how thoroughly they are practiced in the field. The goals for individual LPAI and HPAI control strategies may be different depending on the country, subtype of the virus, economic situation and risk to public health.

There is no single control strategy for AI. In most developed countries, HPAI outbreaks have been eradicated within six months to a year by traditional stamping-out programs, but in some developing countries, the lack of indemnities, poor veteri-

nary infrastructure and high level of poultry production at the village or rural level, have made immediate eradication unachievable. In these situations, management of the disease to a low infection rate has been a realistic option. By comparison, control of LPAI has varied greatly among individual countries, and even between states and provinces within a single country (288). Notably good control programs have emerged from Minnesota (106, 221) and Pennsylvania (41) which have been successful in eradicating LPAI viruses on multiple occasions. Recommendations and responsibilities for containing influenza outbreaks have been described (92). The Minnesota plan has been the model for many other state plans and has five components: education, preventing exposure, monitoring, reporting, and a "responsible response" (221). The designation of H5 and H7 LPAI as LPNAI has increased the use of stamping out programs in dealing with these two AI subtypes as a means to prevent emergence of HPAI viruses. Historically HPAI viruses have emerged after LPAI H5 or H7 viruses circulated widely in susceptible poultry for several months.

Education

One critical aspect in control is the education of all poultry and allied industry personnel regarding how the viruses are introduced, how they spread, and how such events can be prevented. An individual's control of risky behaviors greatly reduces the spread of AI virus by controlling fomite or aerosol movement of the virus thus preventing AI virus movement on the farm and between farms.

Biosecurity

Biosecurity is the first line of defense (see Chapter 1) and is practiced as inclusion biosecurity, such as quarantine, to keep the virus on infected premises and exclusion biosecurity to keep the virus off of virus free premises. The most likely source of virus for poultry is other infected birds, so the basic means for the prevention of infection of poultry with influenza viruses is the separation of susceptible birds from infected birds and their secretions and excretions. Transmission can occur when susceptible and infected birds are in close contact or when infectious material from infected birds is introduced into the environment of susceptible birds. Such introductions are associated with the movement of cages, equipment, footwear and clothing, vehicles, insemination equipment, etc. The presence of virus in fecal material and respiratory secretions is a likely means for movement of the virus either by ingestion, contact with mucous membranes, or inhalation. Contaminated poultry manure is a high risk source for virus transmission between flocks. Certain things have been identified that contribute to spread after AI has been introduced into commercial flocks: unclean moving equipment and crews, partial flock marketing, marketing an actively infected flock, shared rendering pick-up of daily mortality, moving the birds, and inadequate cleaning and disinfection (106). Poultry raised outdoors or that have outdoor access have been infected following exposure to wild birds, primarily to infected ducks and shorebirds. In some countries, LPM and village poultry are an important reservoir of influenza virus and pose a risk for introduction to commercial poultry if adequate biosecurity is not practiced. Swine may serve as a source of H1 and H3 swine influenza viruses to turkeys where the virus is transmitted mechanically or by infected pigs (80).

All biosecurity practices limit spread of influenza by preventing contamination; controlling the movement of birds or their products, people, and equipment; or reducing the amount of the virus (e.g., cleaning and disinfection) (106). Persons who have direct contact with birds or their manure have been the cause of most virus transmission events between houses or premises, but airborne transmission has served as a source to some farms in association with certain depopulation and cleaning activities during the peak of infection (37,69,233). Equipment that comes in direct contact with birds or their manure should not be moved from farm to farm without adequate cleaning and disinfection, and it is important to keep the traffic area near the poultry house free from contamination by manure. Visitors on farms should not be allowed or should be strictly controlled with mandatory disinfection of footwear and cleaning of clothing. Farm-to-farm spread of influenza virus must first be brought under control before the disease can be eradicated.

Special biosecurity procedures must be used when depopulating or marketing infected or dangerous-contact flocks, including re-routing trucks from infected farms away from other poultry farms and the sealing, cleaning, and disinfection of depopulation trucks before they leave farms. In addition, special biosecure practices are needed in repopulating within an infected zone or compartment during the recovery phase to prevent resurgence of the virus.

Diagnostics and Surveillance

Accurate and rapid diagnosis of AI is a prerequisite to early and successful control. The speed with which AI is controlled is largely dependent upon how rapid the first case or cases are detected, the existing biosecurity, and how quickly control strategies are implemented, especially if eradication is the goal. Passive surveillance is critical to differentiate LPAI virus as the cause of respiratory disease or drops in egg production from causes of endemic diseases with similar signs. Similarly, HPAI virus must be differentiated from other causes of high mortality events. Active surveillance is essential to determine where the virus is located within a country, zone, or compartment and can best be accomplished through either serological testing of birds for antibodies and/or random testing of daily mortality for the presence of AI virus. Surveillance is also crucial for on-going evaluation of the success of control strategies and for use in decision making as a prelude to improving control strategies. Serological testing has been used to certify a country, zone, or compartment as AI free, or during an AI outbreak to determine the extent of the infected zone for quarantine purposes.

Elimination of Infected Poultry

After identification of infected flocks, elimination of infected flocks, their eggs, and manure is essential to preventing future transmission. For HPAI, this has been typically accomplished through depopulation and disposal of carcasses, eggs, and manure by an environmentally sound method such as composting,

incineration, rendering, or landfill burial. For LPAI, orderly marketing of birds after recovery from infection has been an acceptable means for elimination, and eggs have been marketed if properly cleaned. Most influenza virus shedding occurs during the first 2 weeks of infection and usually by 4 weeks after the initiation of the infection, virus cannot be detected by sampling. Sero-positive flocks have not been associated with a high risk of transmission if maintained under biosecure practices. However, there should be no contact with recovered flocks because the length of time birds within a population shed virus is not clearly defined. Because the economic losses due to influenza may be severe, the control program should not unnecessarily penalize the growers. Indemnities by federal governments may be necessary for control and eradication of both HPNAI and LPNAI.

Decreasing Host Susceptibility

If poultry are at risk to AI virus exposure, increasing the resistance of birds to infection may be necessary to break the infection cycle. Theoretically, this can be achieved by genetic selection for resistant bird strains or breeds, but to date, only minor chicken breed resistant to LPAI virus has been identified and scientifically verified (301). Another method to increase resistance is through active or passive immunity to the AI viral hemagglutinin or neuraminidase proteins. This is predominantly done through vaccination, but antibody and immune cell transfer can be protective.

Vaccination

Various vaccine technologies have been developed in the laboratory and have shown efficacy in experimental studies, in mostly chickens and turkeys, to provide protection from LPAI and HPAI viruses (284). The most frequently licensed AI vaccine technology has been inactivated whole AI virus vaccines, typically made using LPAI field outbreak strains, and more recently reverse genetic generated AI vaccine strains, followed by chemical inactivation and oil emulsification (285). These vaccines have been used in a variety of poultry and other avian species, and their effectiveness in preventing clinical signs and mortality is well documented. However, protection is virus-subtype specific. Birds are susceptible to infection with influenza viruses belonging to any of the 16 hemagglutinin subtypes, and there is no way to predict their exposure to any particular subtype. It is not practical to use preventive vaccination against all possible subtypes. However, if a particular hemagglutinin subtype is at risk for introduction or after an outbreak occurs and the hemagglutinin subtype of the virus is identified, vaccination may be a useful tool in a control program (111). At this time, there is no substantive data to indicate vaccines based on conserved AI proteins (such as nucleoprotein, matrix proteins, or polymerase proteins) will provide any practical field protection.

Inactivated H5 and H7 AI vaccines, including those in the USDA Vaccine Bank, and a fowl pox-AI hemagglutinin (H5) recombinant vaccine (rFP-AI-H5) are licensed in the United States for potential emergency use in future HPAI or LPNAI eradication efforts. In addition, a conditional license has been granted for other LPAI viral hemagglutinin subtypes (non-H5 and non-H7) of inactivated AI vaccines for limited use, particularly in turkeys (111,175). Numerous experimental studies (15,20,40,43,44,133, 140,270,271,338,350) have demonstrated that AI vaccines can induce antibody and provide protection against mortality, morbidity, and declines in egg production. In addition, properly administered vaccines increase resistance to AI virus infection, reduce the number of birds shedding virus, greatly reduce the titer of challenge virus shed, and prevent contact transmission. Carefully controlled use of vaccines in a H5 and H7 LPNAI outbreak may delay and reduce the chance of the emergence of HPAI viruses. Most frequently, inactivated vaccines are administered prior to anticipated exposure by subcutaneous administration. Chickens can be immunized successfully by the *in ovo* administration of inactivated oil emulsion vaccine (269). The rFP-AI-H5 vaccine is given by subcutaneous or wing web inoculation at 1 day post-hatch in chickens only. The rFP-AI-H5 vaccines cannot be used in chickens that have received a prior pox virus vaccine or have been infected by a field strain of fowl pox virus or AI protection will be inconsistent (294). In the case of many LPAI outbreaks in the United States, producers have been allowed to use USDA licensed inactivated AI vaccines following state veterinarian approval. The limitation of vaccination in this situation is that serologic surveillance is impeded, and viral infection can occur and persist in the absence of disease. However, circulation of natural infection with LPAI viruses in a poultry industry can also impede detection of HPAI infected flocks. To counter the problem, surveillance must be designed and used to identify infected birds within a vaccinated population (i.e., DIVA strategy). One method is surveillance for active infection by detection of AI virus among the daily mortality using antigen capture immunoassay or RRT-PCR. Another method is serological surveillance for antibodies in vaccinated flocks either using routine serological tests (AGID, ELISA, or HI) in non-vaccinated sentinel birds or use of appropriate serological tests for vaccinated birds. For example, with birds vaccinated with an inactivated vaccine, detection of antibodies to the NS1 protein (321) may be used, or if using a heterologous NA vaccine, detection of anti-NA antibodies against the NA of the field AI virus (53) is indicative of infection in vaccinated birds. If using recombinant vaccines with only the AI virus hemagglutinin (such as rFP-AI-H5), detection of antibodies against AI virus NP or M proteins (AGID or ELISA) indicates infection in vaccinated chickens. Vaccinated flocks cannot be considered influenza virus-free without adequate surveillance. Vaccinated flocks must be identified and monitored for the presence of AI virus until slaughtered.

Additional considerations that influence decisions on vaccination for H5 or H7 LPNAI viruses have been discussed (27,107, 108). Previously, the lack of a government indemnity program for LPNAI resulted in some industry segments (e.g., egg layers) being subject to severe economic damage from LPNAI virus. By withholding vaccine availability, regulatory agencies have provided the producer with an incentive to intentionally expose his/her flock to reduce the economic impact of LPNAI on egg production or air sac condemnations. Intentional exposure is likely to contribute to the spread of the disease. Controlled, effective vaccine use will reduce the population of susceptible poultry

and reduce the quantity of virus shed if infection occurs. Recent examples where inactivated H5 or H7 vaccine has been used as an aid in controlling LPNAI include Minnesota (108,288), Utah (98,110), Italy (89), and Connecticut (288). A national U.S. program for control of H5 and H7 LPNAI has been approved under the National Poultry Improvement Plan. This plan provides for indemnities, surveillance guidelines, federal-state-industry partnership and the ability to use vaccines under appropriate conditions (http://www.aphis.usda.gov/vs/npip/, lpai_interim_final_rule[1].pdf).

Approaches to AI vaccines, which are alternatives to the use of inactivated virus and rFP-AI-H5 vaccines, are the use of other vectored vaccines (Rous sarcoma virus, vaccinia, infectious laryngotracheitis, Venezuelan equine encephalitis virus, and adenoviruses) or DNA vaccines incorporating hemagglutinin genes, which have provided protection (32,59,71,100,127,154, 189,224,285,317). These different experimental approaches have been used successfully to immunize and protect birds. Hemagglutinin-based vaccines have been shown to provide protection against a broad array of homologous hemagglutinin subtype viruses (147,293,297). Recently developed vaccine technologies show promise for application by mass immunizing methods that are not possible with current AI vaccine technology, such as Newcastle disease virus vectored AI hemagglutinin vaccine (206,285,310), AI-NDV chimera vaccine (206), and others. Recently, NDV-AI-H5 vectored vaccines have been licensed for use in China and Mexico. These vaccines provide dual protection against ND and AI, but, similar to the pox vectored vaccine, preexisting immunity to ND might interfere with AI protection.

It is clear that opportunities to develop a variety of effective vaccines exist. The ensuing debate (27) centers on the role they should play in controlling influenza viruses of varying pathogenicity in different domestic bird populations in different geographic regions. Based on the multitude of influenza A viruses in wild bird populations, it is reasonable to expect that these viruses will continue to cause serious disease problems when introduced into the LPM, rural or village poultry, and commercial poultry industries. Therefore, judicious use of vaccines may be appropriate to reduce influenza transmission and decrease susceptibility of poultry to the viruses, so eradication methods can be implemented before the disease spreads and becomes endemic.

Treatment

Presently, no practical, specific treatment exists for AI virus infections in commercial poultry. Amantadine has been shown experimentally to be effective in reducing mortality (29,73,80, 159,339), but the drug is not approved for food animals, and its use rapidly gives rise to amantadine-resistant viruses. Supportive care and antibiotic treatment have been employed to reduce the effects of concurrent bacterial infections. The use of human anti-influenza drugs is strongly discouraged.

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Chapter 7

Infectious Bursal Disease

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This chapter is an updated version of the chapter originally authored by Drs. Phil D. Lukert and Y. M Saif for the 11th edition of *Diseases of Poultry*.

Introduction

Infectious bursal disease (IBD) is an acute, highly contagious viral infection of young chickens that has lymphoid tissue as its primary target with a special predilection for the bursa of Fabricius (cloacal bursa). It was first recognized as a specific disease entity by Cosgrove (42) in 1962 and was referred to as "avian nephrosis" because of the extreme kidney damage found in birds that succumbed to infection. Since the first outbreaks occurred in the area of Gumboro, Delaware, "Gumboro disease" was a synonym for this disease and is still frequently used. The economic importance of this disease is manifested in two ways. First, some virus strains may cause up to 20% or more mortality in chickens 3 weeks of age and older. The second, and more important, manifestation is a severe, prolonged immunosuppression of chickens infected at an early age. Sequelae that have been associated with immunosuppression induced by the virus include gangrenous dermatitis, inclusion body hepatitis-anemia syndrome, *E. coli* infections, and vaccination failures. Protection of young chicks from early infection is paramount, and this is usually accomplished by transfer of maternal antibodies to the newly hatched chick. The virus does not affect man and has no public health significance.

History

Early studies to identify the etiologic agent of IBD (avian nephrosis) were clouded by the presence of infectious bronchitis virus in the kidneys of field cases. Winterfield and Hitchner (290) described a virus isolate (Gray) that came from a field case of nephrosis not unlike the newly reported syndrome. Because of the similarity between kidney lesions induced by Gray virus and those seen in avian nephrosis as described by Cosgrove (42), it was believed that Gray virus was the causative agent. Later studies, however, revealed that birds immune to Gray virus could still be infected with the IBD agent and would develop changes in the cloacal bursa specific for the disease. In subsequent studies with IBD, Winterfield *et al.* (291) succeeded in isolating an agent in embryonating eggs. The mortality pattern was irregular, and the agent was difficult to maintain in serial passage. The isolate was referred to as "infectious bursal agent" and was identified as the true cause of IBD; Gray virus was identified as an isolate of infectious bronchitis virus with nephropathogenic tendencies. Hitchner (97) subsequently proposed the term *infectious bursal disease* as the name of the disease causing specific pathognomonic lesions of the cloacal bursa.

In 1972, Allan *et al.* (6) reported that IBD virus (IBDV) infections at an early age were immunosuppressive. The recognition of the immunosuppressive capability of IBDV infections greatly increased the interest in the control of these infections. The existence of a second serotype was reported in 1980 (166). Control of IBD viral infections has been complicated by the recognition of "variant" strains of serotype 1 IBDV, which were found in the Delmarva poultry producing area (220, 227). These strains were breaking through maternal immunity against "standard" strains, and they also differed from standard strains in their biological properties (224, 225). These variants, or subtypes, were either already present in nature but unrecognized or were new mutants that have arisen, possibly due to immune pressure. In the late 1980s, very virulent strains of IBDV (vvIBDV) were isolated in the Netherlands (32), and these strains quickly spread to Africa, Asia, and more recently to South America (52). The vvIBDV strains were not reported from Australia, New Zealand, or the United States.

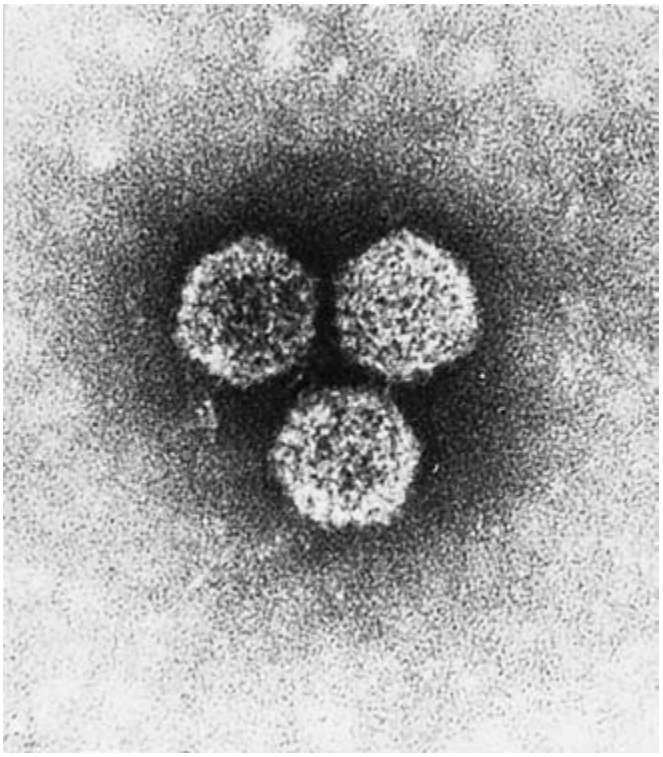
Etiology

Classification

Infectious bursal disease virus is a member of the *Birnaviridae* family (26, 55, 177). The family has 3 genera designated *Aquabirnavirus* whose type species is infectious pancreatic necrosis virus (IPNV), which infects fish, molluscs, and crustaceans; *Avibirnavirus* whose type species is IBDV, which infects birds; and *Entomobirnavirus* whose type species is Drosophila X virus, which infects insects (51). Viruses in that family have genomes consisting of 2 segments of double-stranded RNA (dsRNA) (157, 177, 254), hence the name *birnaviruses*. Before the recognition of the *Birnaviridae* family and before there was adequate information on its morphology and physicochemical characteristics, IBDV was placed at times in the *Picornaviridae* (39, 155) or *Reoviridae* families (83, 133, 151, 205).

Morphology

The virus is a single-shelled, non-enveloped virion with icosahedral symmetry and a diameter varying from 55–65 nm (94, 191, 200) (see Fig. 7.1).



7.1. Electron micrograph of negatively stained infectious bursal disease (IBD) viral particles. ×200,000.

Buoyant density of complete particles in cesium chloride gradients has been reported to range from 1.31–1.34 g/mL (15, 64, 115, 175, 191, 205, 264). Lower density values were reported for incomplete virus particles.

The capsid symmetry is askew, with a triangulation number of T = 13. Once believed to have a capsid with a dextro-handed symmetry (200), IBDV has been shown in recent structural studies to have a typical *laevo* icosahedral geometry (43, 214).

Chemical Composition

The dsRNA of the IBDV genome has two segments designated A and B (15, 55, 116, 177) as shown by polyacrylamide gel electrophoresis. It was reported that the two segments of 5 serotype 1 viruses migrated similarly when co-electrophoresed. The RNA segments from serotype 2 viruses migrated similarly, but differed from serotype 1 viruses when co-electrophoresed (16, 116).

Five viral proteins designated VP1, VP2, VP3, VP4, and VP5 are recognized (15, 53, 55, 182, 191, 264). The approximate molecular weights of the 5 proteins are 97 kD, 41 kD, 32 kD, 28 kD, and 21 kD, respectively. Additional proteins, such as VPX or pVP2, have been observed and have a precursor-product relationship (53). Becht *et al.* (16) compared isolates of serotypes 1 and 2 and reported viral proteins with molecular weights in the same range as those observed by Jackwood *et al.* and Kibenge *et al.* (116, 129). It was not possible to differentiate between strains of serotype 1 viruses based on differences in structural proteins (270). VP2 and VP3 are the major structural proteins of IBDV. In serotype 1 viruses, they constitute 51% and 40% of the virus proteins, respectively (55); whereas VP1 (3%) and VP4 (6%) are minor proteins. However, the exact amount of VP4 in purified IBDV particles is not known precisely, as it was later shown that VP4 is mainly a nonstructural protein that may be copurified with virus particles in cesium chloride gradients because it forms in infected cells type II tubules with the same buoyant density as mature virions (80). In addition to the structural viral proteins, mature virus particles also harbor at their surface four small peptides that are formed when VP2 is progressively matured (47).

VP1 is the viral RNA dependent RNA polymerase (RdRp) and exhibits an original organization as compared with other viral RdRps (77, 282). It is present in virus capsids both as a genome-linked and as a free protein (176). VP2 is the main capsid protein. It forms trimers which are the basic units of the virus shell, the crystal structure of which has been recently determined with a 7 Å resolution (43). VP3, the other major structural protein, interacts with itself, VP2, VP1, and the viral genome, thus playing a critical role in both virion morphogenesis and encapsidation (34, 146, 257). VP3 is most likely not exposed at the surface of the virion, as suggested by the fact that VP2 alone accounts for the capsid crystal structure, but is rather located inside the virus particle in an unordered position—hence undetected by X-ray cristallography (43). VP4 is a viral protease (103, 187) that exhibits an unusual Ser-Lys catalytic dyad (24, 140). VP4 plays a major role in the maturation of capsid protein VP2, by progressively trimming several peptides at the VP2 carboxy-terminal extremity during virus assembly (140). The crystal structure of a birnavirus protease has been recently determined in blotched snakehead virus (69). VP5 has no clearly established function, but it was suggested that it might have a regulatory function playing a role in virus release and dissemination, as well as an anti-apoptotic function at the early stages of infection (144, 147, 184). Two of the peptides that arise from the maturation process of pVP2 are crucial determinants that control the geometry of the virion assembly process (33, 47). One of these peptides also has a destabilizing effect on cellular membranes and has been proposed to be involved, after IBDV particles are bound to their cellular receptor, in the process of virus translocation across the cytoplasmic membrane (33, 47).

The small segment of the IBDV genome (B, approximately 2.9 kbp) codes for VP1, whereas the large segment (A, approximately 3.3 kbp) encodes the VP5 protein and, in another and partially overlapping reading frame, a 110 kDa polyprotein that will yield VP2, VP4, and VP3 upon co-translational cleavage by VP4 (9, 103, 173). A high degree of sequence homology was reported between the pathogenic serotype 1 and the non-pathogenic serotype 2 viruses in the coding region of segment B; whereas lower sequence identities were observed in the coding region of segment A of serotypes 1 and 2 viruses (186). In both genome segments, the coding regions are flanked by short 5′ and 3′ untranslated regions (79 to 111 nucleotide long) (181). The secondary structure of the 3′ untranslated region appears to be critical for an efficient replication (19).

The molecular basis for IBDV antigenicity is now better understood. The VP2 and VP3 proteins were identified in western-

blotting experiments with convalescent sera as important IBDV-derived antigens (64).

VP3 was first believed to represent IBDV major immunogen (65). However, although anti-VP3 neutralizing monoclonal antibodies have been occasionally described (217, 285), it was subsequently shown that VP3 mostly elicits non-neutralizing and non-protective antibodies (16, 67) whereas VP2 is an essential immunogen of IBDV (16, 66). Up to four VP3-located antigenic domains have been identified (122, 159, 198, 300). All contain epitopes common to both serotypes (group-specific epitopes), whereas two of these domains also contain serotype-specific epitopes (159).

Two antigenic domains were also identified in the VP2/pVP2 protein (10). One domain is conformation-independent, located at the carboxy-terminal end of VP2/VPX and elicits non-neutralizing monoclonal antibodies (10, 16, 67). Some of the VP2-specific non-neutralizing epitopes are group-specific (16), whereas others appear to be strain specific (279). The other major VP2 antigenic domain is conformation-dependent, is encoded by the AccI-SpeI fragment (mid-third) of the VP2 gene (10), and mainly groups serotype- or strain-specific epitopes that elicit neutralizing and passively protective antibodies (67, 250). This general pattern is not absolute, though, as Snyder *et al.* reported an anti-VP2 monoclonal antibody that neutralized both serotypes (248, 250). The largest panels of neutralizing monoclonal antibodies (60, 67, 250, 276, 300) detect as many as 6 VP2-located neutralizing epitopes, which co-locate in at least three overlapping antigenic sites. Comparison of the amino acid sequences of several IBDV strains showed that most amino acid changes were encoded within the AccI-SpeI region; this VP2 region has been known since as "VP2 variable domain" (14). Further analysis of IBDV strains with different Mab reactivities identified hot-spots for antigenically significant amino acid changes (61, 89, 137, 229, 272, 276). These are located within stretches of hydrophilic amino acids in the VP2 sequence: aa 212–224 and 314–324 are known as "VP2 major hydrophilic peaks" or "hydrophilic peaks A and B," respectively (229), whereas aa 248–252 and 279–290 are designated as "VP2 minor hydrophilic peaks 1 and 2," respectively (276). Recent structural studies demonstrated these amino acids to be located in the most exposed part of VP2 and to be displayed at the most external surface of the virus particle (43).

The molecular basis for pathogenicity of the virus has not been determined. The development of a reverse genetics system by Mundt and Vakharia (185) made it possible to manipulate the virus allowing for a better understanding of the molecular basis of important biologic activities of the virus. Using this approach, segment A was demonstrated to form the genetic basis for bursal tropism in serotype 1 IBDV (305). A genetically engineered chimeric virus expressing the VP2 gene of a vvIBDV within the genetic context of a classical serotype 1 virus was also shown not to exhibit the very virulent phenotype, thus demonstrating that VP2 is not the sole determinant of virulence (20). Some epidemiological and experimental studies further suggested that both genome segments might be required for the expression of the vvIBDV phenotype (22, 99, 105, 141, 143). It has been suggested that reassortment phenomena might be involved in the emergence of vvIBDV (27, 99).

The chemistry of the virus was reviewed some years ago by Kibenge *et al.* (127).

Virus Replication

Kibenge *et al.* (127) and Nagarajan and Kibenge (186) reviewed this subject some years ago. Some progress has been made in the understanding of the biochemical events associated with replication of birnaviruses. Several laboratory hosts for IBDV are described later in this chapter. The virus was shown to attach to chicken embryo kidney cells maximally 75 minutes after inoculation (151). The multiplication cycle in chicken embryo cells is 10–36 hours, and the latent period is 4–6 hours (15, 116, 151, 191). In Vero and BGM-70 cells, a longer (48-hour) multiplication cycle was described (119, 128, 154).

The cell receptor of the virus is not known. Nieper and Müller suggested that serotype 1 and serotype 2 IBDV may use several receptors on different cell types, these receptors being either common to both serotypes or serotype specific (192). Ogawa *et al.* demonstrated that the receptor for virulent IBDV is a N-glycosylated membrane protein expressed in the IgM-bearing immature B lymphocytes (194). Translocation across the cytoplasmic membrane of the receptor-bound IBDV particles could be mediated by pep46, one of the VP2-derived peptides that are present in the virus particle (33, 47).

The mechanism of viral RNA synthesis has not been clearly determined. A dsRNA-dependent RNA polymerase, VP1, was described (253, 282). Genome-linked proteins have been demonstrated, indicating that the virus replicates its nucleic acid by a strand displacement mechanism (253). Von Einem *et al.* showed that a baculovirus expressed IBDV RdRp specifically used the 3′ untranslated region of an IBDV positive strand template to initiate the synthesis of a complementary strand by a "copy-back" mechanism (282). RNA polymerase activity could be demonstrated without the pretreatment of the virus, indicating that transcription and replication occur following cell penetration without the uncoating of the virus (253). It has been hypothetized that non-polyadenylated mRNAs are extruded through pores possibly located at the 5-fold symetrical axis in IBDV capsid (43).

Becht (15) reported that synthesis of host proteins is not shut off in chicken embryo fibroblasts (CEFs) infected with IBDV. In chicken bursal lymphoid cells grown *in vitro,* the viral polypeptides were detected in the cells and their culture media at 90 minutes and 6 hours post-infection, respectively (175). Tacken *et al.* demonstrated an interaction between VP1 and eukaryotic translation initiation factor 4AII; this association suggests that VP1 could also be involved in the translation of IBDV RNA (258). The lack of accumulation of the polyprotein in infected cells suggests its co-translational cleavage (175). The model currently proposed for the assembly of IBDV particles involves most virus proteins: VP1 most probably first interacts with virus RNA, as described in IPNV, another birnavirus (54). VP3 then interacts with itself, pVP2, VP1 and the viral genome, thus playing a critical chaperone role in virion morphogenesis and encapsidation (34, 146, 257). The final maturation of pVP2 by serial cleavage

of its last carboxy-terminal peptides occurs within the virus capsid (33, 35).

Virus particles accumulate within the cytoplasm of infected cells (161). This could be favored by the fact that VP5 prevents apoptosis at the early stages of infection, by interfering with the caspases and NF-KB pathways (144). However, VP5 (301) and/or VP2 (70) have also been reported to induce apoptosis in infected cells. The VP5-mediated formation of pores in the membrane of the infected cells (147), would contribute to virus release.

Susceptibility to Physical and Chemical Agents

Infectious bursal disease virus is very stable. Benton *et al.* (17) found that IBDV resisted treatment with ether and chloroform was inactivated at pH 12 but unaffected by pH 2, and was still viable after 5 hours at 56°C. The virus was unaffected by exposure for 1 hour at 30°C to 0.5% phenol and 0.125% thimerosal. There was a marked reduction in virus infectivity when exposed to 0.5% formalin for 6 hours. The virus was also treated with various concentrations of three disinfectants (an iodine complex, a phenolic derivative, and a quaternary ammonium compound) for a period of 2 minutes at 23°C. Only the iodine complex had any deleterious effects. Landgraf *et al.* (138) found that the virus survived 60°C but not 70°C for 30 minutes, and 0.5% chloramine killed the virus after 10 minutes. Invert soaps with 0.05% sodium hydroxide either inactivated or had a strong inhibitory effect on the virus (236). Alexander and Chettle (5) detected a biphasic drop in infectivity of the virus in bursal homogenates at 70°, 75°, and 80°C, with initial rapid drop followed in the second phase with a gradual decline. A drop of 1 log 10 at 70°, 75°, and 80°C took 18.8, 11.4, and 3.0 minutes, respectively. Mandeville *et al.* (162) inoculated chicken parts or chicken products with the virus and then cooked them to an internal temperature of 71° and 74°C, respectively, followed by cooling and viable virus was recovered from both products.

Certainly, the hardy nature of this virus is one reason for its persistent survival in poultry houses even when thorough cleaning and disinfection procedures are followed.

Strain Classification

A variety of phenotypic and molecular genetic procedures have been developed and used to classify isolates of IBDV. Classification systems based on phenotypic traits, such as serotyping, have been used successfully since the discovery of the virus. Serotyping of IBDV isolates using polyclonal antibodies in cross virus neutralization (VN) tests has correlated very well with protection studies. The newer molecular genetic procedures have proved extremely useful for diagnostics and epidemiologic studies, but the use of these procedures at this point for classification of isolates has caused some confusion, mostly because of the lack of documented criteria for interpretation of the results and the lack of correlation between serogrouping and molecular grouping. Following are some of the procedures used to classify IBDV isolates.

Antigenicity

McFerran *et al.* (166), in Northern Ireland, were the first to report antigenic variations among IBDV isolates of European origin. They presented evidence for the existence of two serotypes, designated 1 and 2, and showed only 30% relatedness between several strains of serotype 1 and the designated prototype of that serotype. Similar findings were reported in the United States (115, 150), and the American serotypes were designated I and II. Later studies (167) indicated the relatedness of the European and American isolates of the second serotype, and use of the Arabic numerals 1 and 2 to describe the two serotypes of IBDV has been used since. Antigenic relatedness of only 33% between 2 strains of serotype 2 was reported (167), indicating an antigenic diversity similar to that of serotype 1 viruses.

The 2 serotypes are differentiated by virus-neutralization (VN) tests, but they are not distinguishable by fluorescent antibody tests or enzyme-linked immunosorbent assay (ELISA). Immunization against serotype 2 does not protect against serotype 1. The reverse situation cannot be tested because no virulent serotype 2 viruses are available for challenge (108, 118). The first isolates of serotype 2 (115) originated from turkeys, and it was thought that this serotype was host specific. Later studies showed, however, that viruses of serotype 2 could be isolated from chickens (109), and antibodies to serotype 2 IBDVs are common in both chickens and turkeys (113, 227).

Variant viruses of serotype 1 were described (221, 227). Vaccine strains available at the time they were isolated did not elicit full protection against the variants, which are antigenically different from the standard serotype 1 isolates. Jackwood and Saif (114) conducted a cross-neutralization study of 8 serotype 1 commercial vaccine strains, 5 serotype 1 field strains, and 2 serotype 2 field strains. Six subtypes were distinguished among the 13 serotype 1 strains studied. One of the subtypes included all of the variant isolates. Snyder *et al.* (249, 250), using monoclonal antibodies, suggested that a major antigenic shift in serotype 1 viruses had occurred in the field. Sequencing studies later identified several amino acid changes in "VP2 hydrophilic peaks" that correlated with the antigenic changes observed in the variant viruses (89, 137, 272). Sapats and Ignjatovic (230) similarly reported Australian antigenic variants. Australian variants seem to be generated by a similar genetic mechanism as the North American variants, but they differ genetically and antigenically—as detected with monoclonal antibodies—from these. The impact of antigenic variation on cross protection has been less documented for Australian variants.

The vvIBDV strains that were first described in Europe (32) were shown to be mostly antigenically similar to the classic serotype 1 viruses (2, 23, 59, 267, 275, 279). However, further characterization with monoclonal antibodies demonstrated one modified neutralizing epitope in typical vvIBDV, as compared with classic serotype 1 viruses (60). Some atypical vvIBDV with more extensive epitope changes have been occasionally identified (61, 62). Sapats *et al.* recently developed a chicken recombinant antibody (laboratory produced synthetic antibody derived from the lymphocytes of chickens immunized with vvIBDV, same use as monoclonal antibody) that positively recognizes vvIBDV (231).

In summary, there are currently 3 well-documented antigenic types. These are classic (often called standard) and variant

serotype 1 (possibly including an American and an Australian group) and serotype 2 viruses. Subtypes of the three antigenic types have also been described.

Immunogenicity or Protective Types

The term protective type was coined by Lohr (145) to describe a practical procedure to classify infectious bronchitis viruses (IBV) based on their protective potential. Classification of IBV has been problematic. This classification is based on cross protection studies in live birds. As indicated earlier, cross-challenge studies with IBDV has yielded results similar to those obtained by cross VN studies used for the antigenic classification (107). There are two serotype 1 protective types, classic/standard and variant groups. Serotype 2 viruses do not protect against challenge with serotype 1 viruses.

Molecular Genetic Types and Gene Sequencing

Molecular genetic techniques are increasingly used to group different isolates of IBDV (120). These techniques have become popular because of their sensitivity, the time they save, the ability to use them on crude samples or inactivated samples, and they do not require replication of the virus. The most commonly used procedure is the reverse transcriptase/polymerase chain reaction-restriction enzyme fragment length polymorphisms RT/PCR-RFLP, mostly applied to the characterization of the genomic region encoding "VP2 variable domain" (see above). A RT-PCR/RFLP approach has also been implemented for the molecular grouping of IBDV strains according to their segment B restriction profile (263). Currently described molecular groups do not always correspond to antigenic or protective groups, and one has to be careful in interpreting the significance of this classification.

A more thorough molecular characterization can be achieved by sequencing the virus genome and studying the phylogenetic relationships of the studied isolate with reference viruses. Care should be taken that for an optimum assessment of genetic relatedness both genome segments should be characterized (141). As no genetic marker for virulence has yet been defined, attempts to infer the phenotype from genetic data should still be considered as tentative.

Pathogenicity

Chickens are the only animals known to develop clinical disease and distinct lesions when exposed to IBDV. Care should be taken, when comparing experiments aimed at assessing the pathogenicity of different IBDV isolates, that these experiments do include relevant control IBDV strains with a well characterized pathogenicity. Major variables to be standardized in comparative trials are the breed or genetic lineage, age and immune status of the challenged chickens, the dose and route of inoculation of the challenge virus and the possible presence of contaminating viruses in the inoculum (294). Field viruses exhibit different degrees of pathogenicity in chickens. In the authors experience based on the experimental reproduction of acute IBD in specific pathogen free white leghorn chickens, "variant" IBDV induce little if any clinical signs and mortality, but marked bursal lesion, classical IBDV induce approximately 10–50% mortality with typical signs and lesions and vvIBDV induce approximately 50–100% mortality with typical signs and lesions (294). Comparative studies show that it may prove difficult to define cut off values, and that putative vvIBDV, when identified by the genetic sequence of their segment A only, may greatly vary in pathogenicity (141, 278). Vaccine viruses also have varying pathogenic potential in chickens, as discussed later in this chapter.

There has been interest in studying the potential pathogenicity of viruses belonging to serotype 2 in chickens and turkeys. Jackwood *et al.* (118) reported a lack of clinical signs and either gross or microscopic lesions in chickens inoculated with a serotype 2 isolate. Sivanandan *et al.* (240), however, observed typical IBDV lesions in chickens inoculated with the same isolate. In later studies (108), 5 isolates of serotype 2, 3 of chicken origin and 2 of turkey origin (including the isolate studied by Jackwood *et al.* and Sivanandan *et al.*), were found non-pathogenic in chickens.

In turkey poults inoculated at 1–8 days of age, an isolate of serotype 2 from turkeys failed to cause disease or gross or microscopic lesions in the cloacal bursa, thymus, or spleen (117); however, the virus was infectious, and the poults responded serologically to the infection. Nusbaum *et al.* (193) studied experimental infection in 1-day-old poults with isolates representing serotypes 1 and 2 that originated from turkeys. Virus-infected cells were detected by immunofluorescence in the bursa, thymus, spleen, and the Harderian gland of infected birds, but no clinical disease resulted. Only slight gross changes were observed, and no histologic differences were seen between infected and non-infected birds. In general, the distribution of fluorescing (infected) cells from these tissues seemed to indicate that the majority were not lymphocytes. The number of plasma cells in the Harderian gland was reduced at 28 days of age. As indicated earlier, the effect of the host system on pathogenicity of the virus may be profound (85, 265). In recent studies, the OH strain of serotype 2 virus that was back passaged 5 times in chicken embryos was shown to be pathogenic to the embryos. Nonetheless, that virus was not pathogenic for 2-week-old SPF chickens or turkeys (4).

Laboratory Host Systems

Chicken Embryos

Initially, most workers had difficulty in isolating virus or, if successful, in serially transferring virus using chicken embryos. Landgraf *et al.* (138) reported a typical experience using the allantoic sac route of inoculation. On the first passage, all inoculated embryos died; on the second, 30% died; and on the third, there was no embryo mortality.

Continued studies (97) uncovered 3 factors that could explain these difficulties: 1) Embryonating eggs that originated from flocks recovered from the disease were highly resistant to growth of the virus; 2) In early virus passage, the allantoamnionic fluid (AAF) had a very low virus content and the chorioallantoic membrane (CAM) and embryo each had a much higher and nearly equal virus content; 3) Comparison of the allantoic sac, yolk sac, and CAM as routes of inoculation showed the allantoic sac to be the least desirable, yielding embryo-infective dose—50% (EID_{50}) virus titers of 1.5–2.0 $\log_{10}$—lower than those obtained

after inoculation by the CAM route. The yolk sac route gave titers that were intermediate.

Winterfield (289) increased virus concentration in the AAF by serial passage in embryonating eggs. Hitchner (97) used isolate 2512, obtained from Winterfield in the 46th embryo passage, to perform a multi-step growth curve study. He found that virus concentration reached a peak 72 hours post-inoculation.

Injection of the virus into 10-day-old embryonating eggs resulted in embryo mortality from days 3–5 post-inoculation. Gross lesions observed in the embryo were edematous distention of the abdominal region; cutaneous congestion and petechial hemorrhages, particularly along feather tracts; occasional hemorrhages on toe joints and in the cerebral region; mottled-appearing necrosis and ecchymotic hemorrhages in the liver (latter stages); pale "parboiled" appearance of the heart; congestion and some mottled necrosis of kidneys; extreme congestion of lungs; and pale spleen, occasionally with small necrotic foci. The CAM had no plaques, but small hemorrhagic areas were observed at times. Lesions induced in embryos by IBDV variants differ from those induced by standard isolates. Splenomegaly and liver necrosis are characteristic of the lesions induced by the variants, but there is little mortality (224). Two vvIBDV strains were reported to constantly induce high mortality in chicken embryos, whereas, a classic strain induced erratic lower mortality (259). Similar to the situation with the classic strains, the CAM was the most sensitive route for infecting chicken embryos with the vvIBDV strains, but the yolk sac route was a good alternative (259).

Cell Culture

Many strains of IBDV have been adapted to cell cultures of chicken embryo origin, and cytopathic effects have been observed. Cell culture-adapted virus may be quantified by plaque assay or microtiter techniques. Rinaldi *et al.* (219) and Petek *et al.* (208) were able to culture egg-adapted strains of IBDV in CEFs, which proved more sensitive to the virus than either embryonating eggs or suckling mice.

Lukert and Davis (151) successfully adapted wild-type virus from infected bursas to growth in cells derived from the chicken embryo bursas. After 4 serial passages in chicken embryo bursa cells, the virus grew in chicken embryo kidney cells and produced plaques under agar. This virus was subsequently propagated in CEFs and used as an attenuated live virus vaccine (241). In addition to cells of chicken origin, the virus has been grown in turkey and duck embryo cells (168), mammalian cell lines derived from rabbit kidneys (RK-13) (219), monkey kidneys (Vero) (94, 154), and baby grivet monkey kidney cells (BGM-70) (119).

Jackwood *et al.* (119) compared three mammalian cell lines (MA-104, Vero, and BGM-70) for their ability to support several strains of IBDV serotypes 1 and 2, including serotype 1 variants. The viruses replicated in the 3 cell lines, but cytopathic effects were most pronounced in the BGM-70 cells. The growth curve of one strain tested in BGM-70 cells was similar to that in CEFs, and VN titers in BGM-70 cultures compared well with those in CEFs.

A continuous fibroblast cell line of Japanese quail origin (QT35) was found to support the replication of IBDV and several other viral pathogens of poultry (44). These viruses, already adapted to tissue culture, produced a cytopathic effect in the quail cells.

Hirai and Calnek (93) propagated virulent IBDV in normal chicken lymphocytes and in a lymphoblastoid B-cell line derived from an avian leukosis virus-induced tumor. The virus would not replicate in 6 T-cell lymphoblastoid cell lines initiated from Marek's disease tumors (MSB-1, RPL-1, GACL-1, JMCL-1, CVCL-1 and GBCL-1). Their work showed that IgM-bearing B lymphocytes were the probable target cells of IBDV. This was subsequently verified in a study on normal lymphocytes of chickens (188). Lymphocytes from the cloacal bursa and thymus were purified and separated into T cells, B cells, and null cells. The B cells bearing surface IgM were susceptible to IBDV, but the T cells and null cells were not.

Müller (174) enriched Ig-bearing cells by rosetting and cell sorting and observed that IBDV replicated preferentially in a population of proliferating cells and that susceptibility did not correlate with expression of immunoglobulins on their surface. A B-lymphoblastoid cell line from a chicken with lymphoid lesions (LSCC-BK3; 92) was found to be superior to CEF, chicken kidney cells, and BGM-70 cells in propagating several attenuated and pathogenic viruses (266).

Isolation of IBDV from field cases of the disease may be difficult. McFerran *et al.* (166) found it very difficult to isolate and serially propagate the virus in cell cultures of chicken embryo origin. Lee and Lukert (139) attempted isolations of IBDV from turkeys and chickens as well as from samples of challenge strains received from other laboratories. Turkey strains (5 of 5) were readily adapted to CEF cells after 3 to 10 blind passages. Only 2 of 9 chicken strains could be adapted to CEF cells; the other 7 strains could be grown only in chicken embryo bursa cells, even after 20 bursal cell passages.

BGM-70 cells were used successfully for isolation of IBDV from the bursas of naturally infected chickens (228). Usually, a cytopathic effect was detected after 2 or 3 blind passages.

One aspect that should be considered concerning *in vitro* replication of the virus is the possibility of development of defective particles. Müller *et al.* (179) reported that serial passages of undiluted virus in chicken embryo cells resulted in fluctuations in infectivity and the development of a stable small-plaque-forming virus that interfered with the replication of the standard virus and favored the generation of defective particles. The defective particles had lost the large segment of dsRNA. Passage of the virus 6 times in BGM-70 cells or CEF resulted in loss of pathogenicity, but similar passages in chicken embryos did not affect the pathogenicity of the virus (85).

Compared to classic and variant strains of serotype 1, adaptation of the vvIBDV viruses to cell culture has been very difficult (2). Site-directed mutagenesis (185) has been used to identify single amino acids that restrict propagation in cell culture (142, 180), but these amino acids could be strain specific. Later, it was reported (3) that adaptation of the virus to BGM-70 cells resulted in a significant reduction in the ability of the virus to replicate in the bursa of Fabricius. Tsukamoto et al (266) reported that LSCC-BK3 cells were superior to BGM-70 cells and CEF in an infectivity assay.

Pathobiology and Epidemiology

Incidence and Distribution

Infections with serotype 1 IBDV are of worldwide distribution, occurring in all major poultry producing areas. The incidence of infection in these areas is high; essentially, all flocks are exposed to the virus during the early stages of life, either by natural exposure or vaccination. Because of vaccination programs carried out by most producers, all chickens eventually become sero-positive to IBDV. Clinical cases are very rare in the United States because infections are either modified by antibodies or are due to variant strains that do not cause obvious clinical disease. These variant strains seem to be the predominate viruses that exist in the United States. Classical viruses and a local type of variants have been reported in Australia (230). In Europe, Africa, Asia, and South America, the vvIBDV strains seem to predominate.

In the United States, it was shown that antibodies to serotype 2 IBDV were widespread in chicken (113, 227) and turkey flocks (11, 37, 115), indicating the common prevalence of the infection.

Natural and Experimental Hosts

Chickens and turkeys are the natural hosts of the virus. A serotype 1 virus was isolated from two 8-week-old ostrich chicks that had lymphocyte depletion in the bursa of Fabricius, spleen, and/or thymus (293). Another serotype 1 isolate was obtained from healthy ducks (166). A serotype 2 IBDV was isolated from captive penguins that died without specific clinical signs (78). Van den Berg (277) inoculated pheasants, partridges, quails, and guinea fowl with vvIBDV. These authors did not report any clinical signs or lesions in these species, however quails replicated the virus in their bursa, shed it for five days in their feces and developed neutralizing antibodies. This contrasts with a previous study by Weisman and Hitchner, who could not infect Coturnix quail with a chicken-origin virus (284), but confirms an earlier report that IBDV-inoculated Guinea fowl did not develop lesions or antibody (196).

Several species of free-living and captive birds of prey were examined for antibodies to IBDV, and positive results were obtained from accipitrid birds (271). Antibodies to IBDV were also detected in rooks, wild pheasants, and several rare avian species (30); in Antartic penguins (74); in ducks, gulls and shearwaters (287); and crows, gulls and falcons (195).

For many years, the chicken was considered the only species in which natural infections occurred. All breeds were affected, and many investigators observed that white leghorns exhibited the most severe lesions and clinical signs and had the highest mortality rate. Meroz (170), however, found no difference in mortality between heavy and light breeds in a survey of 700 outbreaks of the disease.

The period of greatest susceptibility to clinical disease is between 3 and 6 weeks of age. Susceptible chickens younger than 3 weeks do not exhibit clinical signs but have subclinical infections that are economically important as a result of severe immunosuppression of the chicken. This immunosuppressive effect of IBDV was first recognized by Allan *et al.* (6) and Faragher *et al.* (68) and is discussed later in this chapter.

The reason for the apparent age susceptibility of chickens to IBDV has been the subject of several research publications regarding the pathogenesis of IBDV infections. Fadly *et al.* (63) treated 3-day-old chicks with cyclophosphamide and found that they were refractory to clinical signs and lesions when challenged at 4 weeks of age. Kaufer and Weiss (126) found similar results with birds surgically bursectomized at 4 weeks of age. When they were challenged immediately, or 1 week later, there was no clinical disease, whereas 100% of the control non-bursectomized chickens died. Bursectomized chickens challenged with virulent virus produced 1000 times less virus than control birds, produced VN antibodies by day 5, and had only very discrete and transient necrosis of lymphatic tissues.

Several studies on the pathogenesis of IBDV infections have been conducted. Skeeles *et al.* (242) attempted to demonstrate that the hemorrhagic lesions were a result of formation of immune complexes, as proposed by Ivanyi and Morris (111). Histologic lesions in the cloacal bursa resemble an Arthus reaction (necrosis, hemorrhage, and large numbers of polymorphonuclear leukocytes). This reaction is a type of localized immunologic injury caused by antigen-antibody-complement complexes that induce chemotactic factors, which cause hemorrhage and leukocyte infiltration. They found that 2-week-old and 8-week-old chicks produced rapid and high levels of antibody by 72 hours post-infection, but that 2-week-old chickens had very little complement compared with 8-week-old chickens. They postulated that the reason 2-week-old chickens did not develop Arthus-type lesions was a lack of sufficient complement. They also showed that complement was depleted in 8-week-old chickens at 3, 5, and 7 days post-infection compared with uninfected controls. A later study by Skeeles *et al.* (245) with another IBDV isolate failed to substantiate the depletion of complement at 3 days post-infection.

Kosters *et al.* (133) and Skeeles *et al.* (242, 245) found increased clotting times in IBDV-infected chickens and suggested that such coagulopathies would contribute to the hemorrhagic lesions observed with this disease. Skeeles *et al.* (245) found that 17-day-old chickens did not exhibit clotting defects, but at 42 days, they had greatly increased clotting times and became clinically ill; 4 of 11 died. The key to the pathogenesis of IBDV in birds of different ages may lie with the factors involved in the clotting of blood and/or an immunologic injury. The pathogenesis is certainly not straightforward and simple.

Naturally occurring infections of turkeys and ducks by serotypes 2 and serotype 1 viruses, respectively, have been recorded (125, 166, 168, 201). Serologic evidence and isolation of IBDV from these species indicate that natural infections do occur. McNulty *et al.* (168) examined turkey serums from several flocks and could not detect IBDV antibodies prior to 1978, suggesting that IBDV infections of turkeys were a relatively new occurrence. Owoade *et al.* reported the isolation of an IBDV strain with genetic relatedness to vvIBDV in a turkey flock in Nigeria (199).

Giambrone *et al.* (76) found that experimental IBDV infections of turkeys were subclinical in 3- to 6-week-old poults, producing microscopic lesions in the bursa. Virus-infected cells in the bursa were detected by immunofluorescence. Neutralizing

antibody was detected 12 days post-infection, and the virus could be reisolated after 5 serial passages in chicken embryos. Weisman and Hitchner (284) could not reisolate virus from their 6- to 8-week-old IBDV-infected poults, but they observed an increase in VN antibody. Infection was subclinical, and no damage to the bursa was evident.

Transmission, Carriers, and Vectors

Infectious bursal disease is highly contagious, and the virus is persistent in the environment of a poultry house. Benton *et al.* (18) found that houses from which infected birds were removed were still infective for other birds 54 and 122 days later. They also demonstrated that water, feed, and droppings taken from infected pens were infectious after 52 days.

No evidence suggests that IBDV is transmitted through the egg or that a true carrier state exists in recovered birds. Resistance of the virus to heat and disinfectants is sufficient to account for virus survival in the environment between outbreaks. Snedeker *et al.* (246) demonstrated that the lesser mealworm (*Alphitobius diaperinus*), taken from a house 8 weeks after an outbreak, was infectious for susceptible chickens when fed as a ground suspension. In another study (165), the virus was isolated from several tissues of surface-sterilized lesser mealworm adults and larvae that were fed the virus earlier.

Howie and Thorsen (100) isolated IBDV from mosquitoes (*Aedes vexans*) that were trapped in an area where chickens were being raised in southern Ontario. The isolate was non-pathogenic for chickens. Okoye and Uche (197) detected IBDV antibodies by the agar-gel precipitin (AGP) test in 6 of 23 tissue samples from rats found dead on 4 poultry farms that had histories of IBDV infection. There has been no further evidence to support a conclusion that either mosquitoes or rats act as vectors or reservoirs of the virus.

Pagès-Manté *et al.* reported that a dog fed chickens that had died of acute IBD shed viable vvIBDV in its feces for two but not for three days after ingestion (202).

As indicated earlier in this chapter, several avian species were shown to be susceptible to the infection or to have antibodies against the virus.

Incubation Period and Clinical Signs

The incubation period is very short, and clinical signs of the disease are seen within 2–3 days after exposure.

One of the earliest signs of infection in a flock is the tendency for some birds to pick at their own vents. Cosgrove (42), in his original report, described soiled vent feathers, whitish or watery diarrhea, anorexia, depression, ruffled feathers, trembling, severe prostration, and finally, death. Affected birds became dehydrated, and in terminal stages of the disease, had a subnormal temperature.

Morbidity and Mortality

In fully susceptible flocks, the disease appears suddenly, and there is a high morbidity rate, usually approaching 100%. Mortality may be nil but can be as high as 20–30%, exceptionally higher with vvIBDV, usually beginning on day 3 post-infection and peaking and receding in a period of 5–7 days. In the late 1980s, strains of vvIBDV became a problem in Europe. Several of these isolates caused mortality rates of 90% (32) to 100% (275) in 4-week-old susceptible leghorn chickens. A 1970 isolate (52/70) (29) was compared with 2 vvIBDV isolates in a study; it caused 50% mortality compared with 90% for the vvIBDV strains (32). In another such study, the vvIBDV isolates caused lower mortality, however percent mortality was again at least twice as much as caused by the 52/70 isolate (59).

Initial outbreaks on a farm are usually the most acute. Recurrent outbreaks in succeeding broods are less severe and frequently go undetected. Many infections are silent, owing to age of birds (less than 3 weeks old), infection with avirulent field strains, or infection in the presence of maternal antibody.

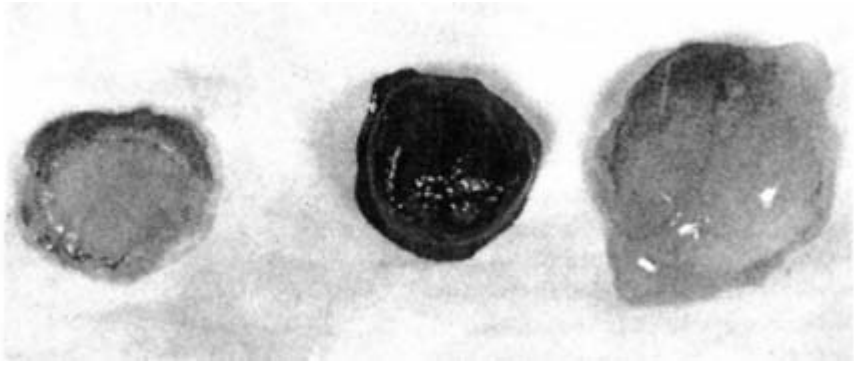


7.2. Hemorrhages of leg muscle typical in IBD.

Pathology

Gross Lesions

Birds that succumb to the infection are dehydrated, with darkened discoloration of pectoral muscles. Frequently, hemorrhages are present in the thigh and pectoral muscles (Fig. 7.2). There is increased mucus in the intestine, and renal changes (42) may be prominent in birds that die or are in advanced stages of the disease. Such lesions are most probably a consequence of severe dehydration. In birds killed and examined during the course of infection, kidneys appear normal.



7.3. Edematous (*right*) and hemorrhagic *(center)* cloacal bursas typical in acute IBD at 72–96 hours post-infection. The bursa on the left is normal.

The cloacal bursa appears to be the primary target organ of the virus. Cheville (36) made a detailed study of bursal weights for 12 days post-infection. It is important that the sequence of changes be understood when examining birds for diagnosis. On day 3 post-infection, the bursa begins to increase in size and weight because of edema and hyperemia (Fig. 7.3). By day 4, it usually is double its normal weight, and the size then begins to recede. By day 5, the bursa returns to normal weight, but it continues to atrophy, and from day 8 forward, it is approximately one-third its original weight, or even less.

By day 2 or 3 post-infection, the bursa has a gelatinous yellowish transudate covering the serosal surface. Longitudinal striations on the surface become prominent, and the normal white color turns to cream color. The transudate disappears as the bursa returns to its normal size, and the organ may become gray during and following the period of atrophy.

Isolates of variant IBDV were reported not to induce an inflammatory response (221, 234), although one variant strain (IN) did so (86).

The infected bursa often shows necrotic foci and at times petechial or ecchymotic hemorrhages on the mucosal surface. Occasionally, extensive hemorrhage throughout the entire bursa has been observed (see Fig. 7.3); in these cases, birds may void blood in their droppings.

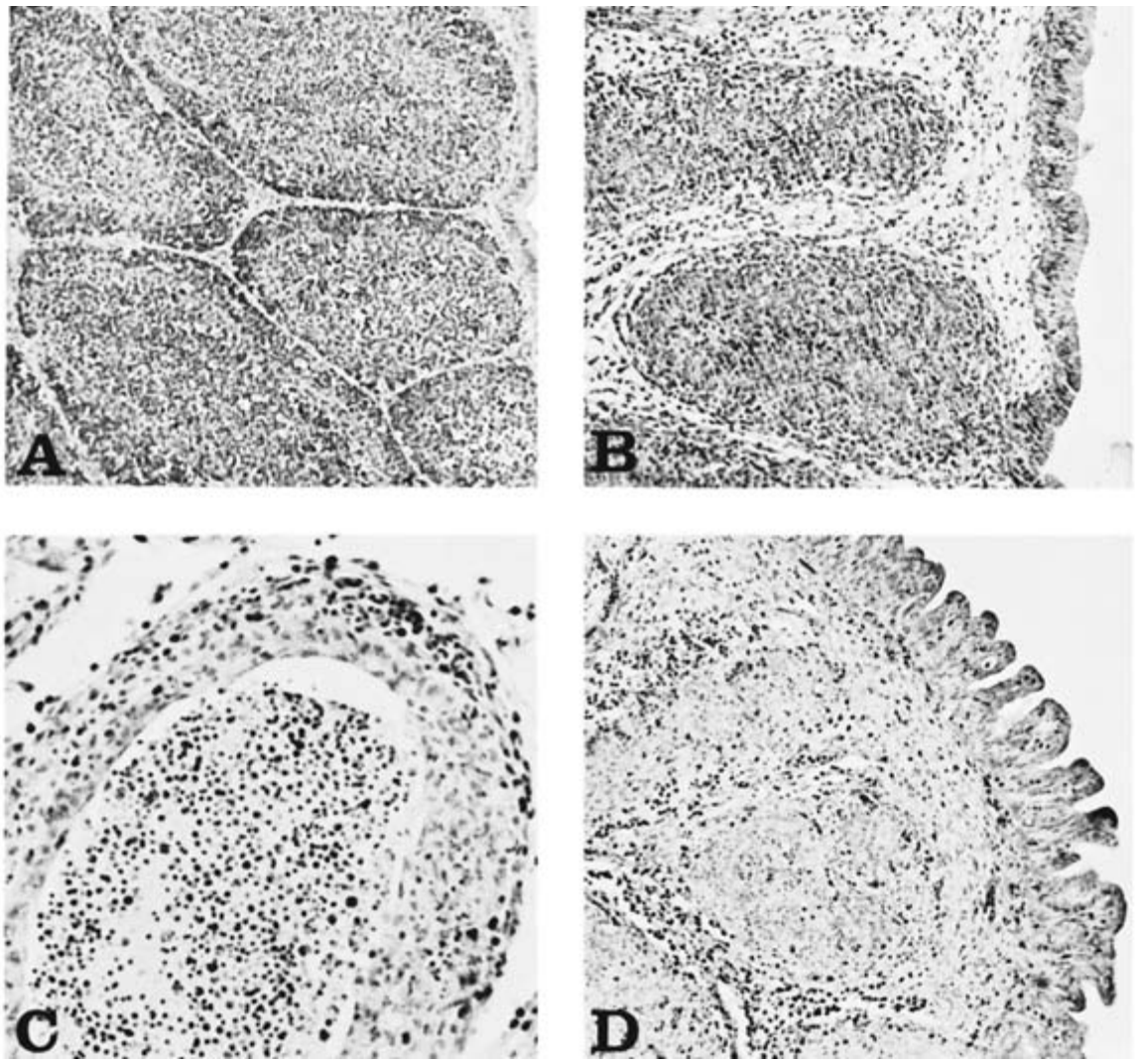
The spleen may be slightly enlarged and very often has small gray foci uniformly dispersed on the surface (218). Occasionally, hemorrhages are observed in the mucosa at the juncture of the proventriculus and gizzard.

Compared with a moderately pathogenic strain of the virus, the vvIBDV strains caused a greater decrease in thymic weight index and more severe lesions in the cecal tonsils, thymus, spleen, and bone marrow, but bursal lesions were similar. It was also shown that pathogenicity correlated with lesion production in non-bursal lymphoid organs, suggesting that pathogenicity may be associated with antigen distribution in non-bursal lymphoid organs (260).

Microscopic Lesions

Microscopic lesions of IBD occur primarily in the lymphoid tissues (i.e., cloacal bursa, spleen, thymus, Harderian gland, and cecal tonsil). Histopathology at the level of light microscopy has been originally studied by Helmboldt and Garner (91), Cheville (36), Mandelli *et al.* (161), and Peters (209). Changes were most severe in the cloacal bursa. As early as 1 day post-infection, there was degeneration and necrosis of lymphocytes in the medullary area of bursal follicles. Lymphocytes were soon replaced by heterophils, pyknotic debris, and hyperplastic reticuloendothelial cells. Hemorrhages often appeared but were not a consistent lesion. All lymphoid follicles were affected by 3 or 4 days post-infection. The increase in bursal weight seen at this time was caused by severe edema, hyperemia, and marked accumulation of heterophils. As the inflammatory reaction declined, cystic cavities developed in medullary areas of follicles; necrosis and phagocytosis of heterophils and plasma cells occurred; and there was a fibroplasia in interfollicular connective tissue. Proliferation of the bursal epithelial layer produced a glandular structure of columnar epithelial cells containing globules of mucin. During the suppurative stage, scattered foci of lymphocytes appeared but did not form healthy follicles during the observation period of 18 days post-inoculation (91). Some of the histologic changes observed in the cloacal bursa are shown in Figure 7.4. A recent isolate (variant A) of IBDV was reported to cause extensive lesions in the cloacal bursa, but an inflammatory response was lacking (234).

A recent sequential study (292) of the recovery of the cloacal bursa after neonatal infection by classical IBDV demonstrated that the initial depletion of B lymphocytes was maximum during the first week, and combined with a transient massive influx of T cells and macrophages until three days post-infection. From 1 to 8 weeks post-infection, two distinct types of bursal follicles were then observed: large reconstituted functional follicles, most likely reconstituted from endogenous bursal stem cells that survived IBDV infection, and small poorly developed follicles lacking a discernible cortex and medulla. The structure of these small follicles suggested they were unable to produce functional peripheral B cells, which hypothesis was confirmed by the lack of active antibody responses in birds lacking large reconstituted follicles. After their initial influx in the bursa, T cells declined in number along with viral clearance and persisted mostly in the small follicles during the recovery phase. Inflammatory foci persisted during the recovery phase, possibly centered on antigen presenting cells. It is not known whether their presence was associated with persistent IBDV infection in some cells (292). Essentially similar but more severe and more persistent histolog-



7.4. Photomicrographs of 6-week-old birds affected with infectious bursal disease virus (IBDV). Tissues are cloacal bursa fixed in 10% buffered formalin and stained with H & E. A. Normal tissue. Large active follicles consist of lymphoid cells that form discrete follicles with little interfollicular tissue. Covering epithelium is simple columnar. ×40. B. Bursa approximately 24 hours post-infection. Note interfollicular edema mixed with phagocytic cells, many of which are heterophils. Follicles are already beginning to degenerate. ×40. C. Single follicle approximately 60 hours post-infection. Medullary portion is now a mass of cellular debris surrounded by cortical remnants. Only reticular cells exist in any number, but scattered among them are a few lymphocytes that will later regenerate. ×250. D. Terminal phase of severe infection. Only ghosts of follicles remain, and heterophils (scattered dark cells) are actively engaged in phagocytosis. Covering epithelium is plicated and grandular, with globules of mucins. ×40. (91).

ical lesions have been described following challenge with a vvIBDV, which however also induced thymic lesions (288).

The spleen had hyperplasia of reticuloendothelial cells around the adenoid sheath arteries in early stages of infection. By day 3, there was lymphoid necrosis in the germinal follicles and the periarteriolar lymphoid sheath. The spleen recovered from the infection rather rapidly, with no sustained damage to the germinal follicles.

The thymus and cecal tonsils exhibited some cellular reaction in the lymphoid tissues in early stages of infection, but, as in the spleen, the damage was less extensive than in the bursa, and recovery was more rapid. A variant virus (A) was reported to cause milder lesions in the thymus than a standard isolate (IM) (234).

Survashe *et al.* (256) and Dohms *et al.* (57) found that the Harderian gland was severely affected following infection of 1-day-old chicks with IBDV. Normally, the gland is infiltrated and populated with plasma cells as the chicken ages. Infection with IBDV prevented this infiltration. From 1–7 weeks of age, the glands of infected chickens had populations of plasma cells 5–10-fold fewer than those of uninfected controls (57). In contrast, broilers inoculated with IBDV at 3 weeks of age had plasma cell necrosis in the Harderian gland 5–14 days post-inoculation, and the plasma cells were reduced by 51% at 7 days after inocu-

lation (58). Reduction in plasma cells, however, was transient, and the numbers were normal after 14 days.

Histologic lesions of the kidney are non-specific (209) and probably occur because of severe dehydration of affected chickens. Helmboldt and Garner (91) found kidney lesions in less than 5% of birds examined. Lesions observed were large casts of homogeneous material infiltrated with heterophils.

The liver may have slight perivascular infiltration of monocytes (209).

Ultrastructural

Naqi and Millar (189) followed the sequential changes in the surface epithelium of the cloacal bursa of IBDV-infected chicks by scanning electron microscopy. They observed a reduction in number and size of microvilli on epithelial cells at 48 hours post-inoculation. There was gradual loss of the button follicles normally seen at the surface, and by 72 hours, most had involuted. By 96 hours, there were numerous erosions of the epithelial surface. The surface was intact by day 9 post-inoculation, but follicles were involuted, leaving deep pits.

Pathogenesis of the Infectious Process

Helmboldt and Garner (91) detected histologic evidence of infection in the cloacal bursa within 24 hours. In sequential studies of tissues from orally infected chickens using immunofluorescence, viral antigen was detected in macrophages and lymphoid cells in the cecum at 4 hours after inoculation; an hour later, virus was detected in lymphoid cells in the duodenum and jejunum (178). The virus first reaches the liver, where it is detected 5 hours post-inoculation. It then enters the bloodstream, where it is distributed to other tissues including the bursa; the bursal infection is followed by a second massive viremia, however virus peak titer in the non-lymphoid organs is several log10 lower than in the bursa and limited to the viremic period.

Studies of gene expression during acute IBD demonstrated the activation of spleen macrophages (130) and of bursal T cells (131). Ruby *et al.* recently provided an extensive transcriptional study (226) of the early host responses to neonatal infection with a classical IBDV (F52/70). Changes in the expression levels of bursal genes closely mirrored the early histological changes discussed earlier in this chapter: low level of expression of B-cell-specific genes in relation with B-cell depletion, enhanced expression of numerous genes related with the activation of macrophages, T cells, and NK cells. Early upregulated genes included genes of the antiviral interferon system (IFN α/β and the IFN-induced genes), cytokines (IL-18, IL-6), and chemokines (IL-8, MIP-1β...); genes involved in the innate immune response (MD-1 and MD-2, complement components, heat shock proteins HSP70 and 47); some genes encoding cytoskeleton proteins; and genes involved in both the proinflammatory and inflammatory responses. Interestingly, the latter genes had significantly more expression at 24 hours PI in an IBD-resistant chicken line, as compared with a susceptible one. Another difference in the resistant line was a transient overexpression of p53 at 48 hours PI. On the other hand, a number of genes involved in B and T cell proliferation were down-regulated by IBDV infection. The authors propose that in resistant birds, a more rapid inflammatory response and more-rapid activation activation of apoptotic pathways in IBDV target cells would limit virus replication and its subsequent pathological effects (226).

The virus was shown to persist in bursal tissues of experimentally inoculated SPF chickens up to 3 weeks, but it persisted for shorter periods in the presence of maternal antibodies in commercial broilers (1).

Based on the fact that IBDV-induced pathology still progresses several days after the viral load starts to decline (5 days PI), Williams and Davison suggested that the disease mechanism is related to immunopathology as well as to virus-induced lysis (288).

Immunity

Viruses of both serotypes of IBDV share common group antigen(s) that can be detected by the fluorescent antibody test and ELISA (106, 115). Hence, it is not possible to distinguish serotypes or their antibodies by these tests. The common (group) antigens for both serotypes are on VP2 (40 kD) and VP3 (32 kD). VP2 also has serotype-specific group antigens that induce VN antibodies (10, 16). Becht *et al.* (16) reported that antibodies against VP3 do not have any protective effect. *In vivo* studies (107, 118) corroborated this observation, because chickens having antibodies to serotype 2 viruses were not protected against serotype 1 viruses. The current thought is that VP2 has the major antigens that induce protection (10, 16).

Traditionally, serotype 1 viruses have been used for studies of the immune response to IBDV. All known isolates of serotype 2 were reported to be non-pathogenic in chickens and turkeys (108, 117, 118) or of very low pathogenicity (40, 193, 207). The discovery of variant strains of serotype 1 has heightened interest in furthering the knowledge of the immune response to IBDV. It was interesting that variants were originally isolated from chickens that had VN antibodies to serotype 1 (221, 227). Inactivated vaccines and a live vaccine made from variant strains protected chickens from disease caused by either variant or standard strains, whereas inactivated vaccines made from standard strains did not protect, or only partially protected, against challenge with variant strains (107, 225).

Five different subtypes of serotype 1 IBDV were tested as inactivated vaccines against a variant strain of a different subtype (107). Vaccines made with 10^8 but not 10^5 tissue-culture-infective doses-50% were protective against a challenge dose of 10^2 EID_{50}. Even the higher vaccine dose did not protect against challenge with $10^{3.5}$ EID_{50}. Based on these results, it was suggested that all the subtypes of serotype 1 share a minor antigen(s) that elicits protective antibodies.

The contribution of humoral immunity to protection has been well documented as indicated by protection conferred by passive transfer of antibodies. Evidence is accumulating on the additive effect of cell-mediated immunity in protection from the disease (216, 235). Recent studies indicated the natural resistance of some breeds of chickens to the disease (84).

Active Immunity

Field exposure to the virus, or vaccination with either live or killed vaccines, stimulates active immunity. Antibody response

may be measured by several methods—VN, AGP, or ELISA tests. Antibody levels are normally very high after field exposure or vaccination, and VN titers greater than 1:1000 are common. Adult birds are resistant to oral exposure to the virus but produce antibody after intramuscular or subcutaneous inoculation of IBDV (98). However, partial protection against IBD was achieved in chickens in the absence of detectable neutralizing antibodies, as a result of experimental immunization with a fowlpox recombinant virus that expresses the VP2 protein (13), which finding is an indication that cell-mediated immunity may also play an important role in protecting against IBD.

Passive Immunity

Antibody transmitted from the hen via the yolk of the egg can protect chicks against early infections with IBDV, with resultant protection against the immunosuppressive effect of the virus. The half-life of maternal antibodies to IBDV is between 3 and 5 days (244). Therefore, if the titer of neutralizing antibodies in the progeny is known, the time that chicks will become susceptible can be predicted. Lucio and Hitchner (148) demonstrated that after antibody titers fell below 1:100, chicks were 100% susceptible to infection, and titers from 1:100 to 1:600 gave approximately 40% protection against challenge. Skeeles *et al.* (244) reported that titers must fall below 1:64 before chickens can be vaccinated effectively with an attenuated strain of IBDV. These figures should be considered as indicative that significant differences have been reported in the neutralizing titers determined by different laboratories (169). Use of killed vaccines in oil emulsions (including variant strains) to stimulate high levels of maternal immunity is extensively practiced in the field. Studies by Lucio and Hitchner (148) and Baxendale and Lutticken (12) indicated that oil emulsion IBD vaccines can stimulate adequate maternal immunity to protect chicks for 4–5 weeks, and progeny from breeders vaccinated with live vaccines are protected for only 1–3 weeks. As with many diseases, passively acquired immunity to IBDV can interfere with stimulation of an active immune response.

Immunosuppression

Allan *et al.* (6) and Faragher *et al.* (68) first reported immunosuppressive effects of IBDV infections. Suppression of the antibody response to Newcastle disease virus was greatest in chicks infected at 1 day of age. There was moderate suppression when chicks were infected at 7 days, and negligible effects when infection was at 14 or 21 days (68). Hirai *et al.* (95) demonstrated decreased humoral antibody response to other vaccines as well. Not only was response to vaccines suppressed, but chicks infected early with IBDV were more susceptible to inclusion body hepatitis (63), coccidiosis (7), Marek's disease (38, 232), hemorrhagic-aplastic anemia and gangrenous dermatitis (223), infectious laryngotracheitis (222), infectious bronchitis (206), chicken anemia agent (303), and salmonellosis and colibacillosis (295).

A paradox associated with IBDV infections of chickens is that although there is immunosuppression against many antigens, the response against IBDV itself is normal, even in 1-day-old susceptible chickens (243). It has been suggested that the ability of the chickens to develop an active anti-IBDV antibody response correlates, after challenge at day-old, with the development of large bursal follicles during the recovery phase (292). This may not be the only mechanism, though, as older chickens that did not succumb to infection and exhibit an extreme bursal atrophy often develop high titers of neutralizing antibodies. It should hence be further investigated whether there is a selective stimulation of the proliferation of B cells committed to anti-IBDV antibody production.

The effect of IBD on cell-mediated immune (CMI) responses is transient and less obvious than that on humoral responses. Panigrahy *et al.* (203) reported that IBDV infections at a young age caused a delayed skin graft rejection; however, other workers (75, 102) found no effect from early IBDV infections on skin graft rejection or tuberculin-delayed hypersensitivity reaction. Sivanandan and Maheswaran (239) observed suppression of CMI responsiveness, using the lymphoblast transformation assay. They found that maximal depression of cellular immunity occurred 6 weeks post-infection. Nusbaum *et al.* (193) detected a significant suppression of T-cell response to the mitogen concanavalin A in poults from 3 days to 4 weeks post-infection. There was no reduction, however, in tuberculin reactions in IBDV-infected poults. In a sequential study of peripheral blood lymphocytes from chickens inoculated with IBDV, a transient depression of mitogenic stimulation was reported (41). Sharma and Lee (233) reported an inconsistent effect of IBDV infection on natural killer cell toxicity and a transient early depression of the blastogenic response of spleen cells to phytohemagglutinin. Craft *et al.* (45) demonstrated that a variant IBD virus strain (A) had a significantly more severe effect on the CMI response than a standard strain (Edgar) when given to 1-day-old chicks, and the CMI was suppressed for 5 weeks. A similar transient suppression of the CMI was observed in chickens infected at 3 weeks of age.

Another component of the immune system is the Harderian gland, which is associated with the local immune system of the upper respiratory tract. Pejkovski *et al.* (206) and Dohms *et al.* (57) reported that IBDV infection of 1–5-day-old chicks produced a drastic reduction in plasma cell content of the Harderian gland that persisted for up to 7 weeks. There have been similar observations with IBDV infections of poults (193). In other studies on broilers infected with IBDV at 3 weeks of age, extracts from the Harderian gland and serum had reduced antibody titers to *Brucella abortus* (a T-cell-independent antigen) and sheep red blood cells (SRBC, a T-cell-dependent antigen). Compared with SRBC antibody response, diminished antibody responses to *B. abortus* were evident at a later time period. A variant virus of serotype 1 produced a similar effect in chickens (56).

Chickens infected with IBDV at 1 day of age were completely deficient in serum immunoglobulin G and produced only a monomeric immunoglobulin M (IgM) (110, 111). The number of B cells in peripheral blood was decreased following infection with IBDV, but T cells were not appreciably affected (96, 237). The virus appears to replicate primarily in B lymphocytes of chickens (93, 110, 299). Apparently, IBDV has a predilection for actively proliferating cells (174), and it was suggested that the

virus affected "immature," or precursor, B lymphocytes to a greater extent than mature B lymphocytes (163).

Beside lymphocyte lysis, apoptosis is another mechanism of immunosuppression. Apoptosis is also a mechanism of lesion development and could occur in a variety of tissues and organs (6, 136, 261, 280, 281).

Although evidence is accumulating on the role of T cells in protection, there is also evidence of a role in immunopathogenesis (216, 235) resulting from tissue destruction enhancement mediated by cytokines.

Diagnosis

Acute clinical outbreaks of IBD in fully susceptible flocks are easily recognized, and a presumptive diagnosis can be readily made. The rapid onset, high morbidity, spiking mortality curve, and rapid recovery (5–7 days) from clinical signs are characteristics of this disease. Confirmation of the diagnosis can be made at necropsy by examination for characteristic grossly visible changes in the cloacal bursa. Remember that there are distinctive changes in size and color of the bursa during the course of infection (i.e., enlargement due to inflammatory changes followed by atrophy) (see "Gross Lesions").

Infections of very young chicks, or chicks with maternal antibody, are usually subclinical and are diagnosed retrospectively at necropsy with observations of macroscopic and microscopic bursal atrophy. Infections of chickens of any age with variant strains of IBDV will be detected only by histopathology of the cloacal bursa or by virus isolation.

Isolation and Identification of the Causative Agent

The cloacal bursa and spleen are the tissues of choice for the isolation of IBDV, but the bursa is the most commonly used for it contains the highest virus titers. Other organs contain the virus, but at a lower concentration and probably only because of the viremia. Tissues should be macerated in an antibiotic-treated broth or saline and centrifuged to remove the larger tissue particles. The supernatant fluid then is used to inoculate embryonating eggs or cell cultures.

Hitchner (97) demonstrated that the chorioallantoic membrane (CAM) of 9–11-day-old embryos was the most sensitive route for isolation of the virus. The virus subsequently could be adapted to the allantoic sac and yolk sac routes of inoculation. Death of infected embryos usually occurs in 3–5 days. Variant strains of IBD differ from standard viruses in that they induce splenomegaly and liver necrosis of embryos and produce little mortality (224). The embryonating egg may be the most sensitive substrate for isolation of IBDV. McFerran *et al.* (166) reported that 3 of 7 chicken isolates of IBDV failed to grow in chicken embryo fibroblast (CEF) cells; however, they could be propagated in embryonating eggs.

Isolation and propagation of IBDV in cell culture was discussed previously in this chapter (see "Laboratory Host Systems"). Because the virus has been shown to replicate in B lymphocytes, either primary cells derived from the cloacal bursa or continuous cell lines of B-cell origin would be the cells of choice for the isolation of the virus. It appears that some strains of virus are very fastidious, and although they may replicate in embryonating eggs or B lymphocytes, they cannot readily be adapted to CEF cells or cells from other organs such as the kidney and liver (139, 166). The use of immunofluorescence and electron microscopy of infected embryos and cell cultures has proven to be of tremendous value for the early detection and identification of IBDV. Cell cultures containing 50% bursal lymphocytes and 50% CEF have been used to successfully isolate and serotype IBD viruses (149). The fibroblasts serve as a matrix for the lymphocytes, and the infected lymphocytes are detected by immunofluorescence. BGM-70 cells may also be used for isolation of IBDV.

Identification of the virus by direct immunofluorescent staining of affected organs or direct examination by electron microscopy has proven to be an adjunct to the isolation and identification of IBDV (166). If antigen or virus is detected by these methods from field cases of disease, every effort should be made to isolate the virus using both embryonating eggs and cell-culture techniques. The isolation, antigenic analysis, and pathogenicity studies of viruses from field cases of IBD are needed continually so that changes in the wild virus population can be detected.

Nucleic acid probes (112) and antigen-capture enzyme immunoassays using monoclonal antibodies (248) to detect and differentiate IBD viruses directly in tissues may prove beneficial for rapid diagnosis and typing of field viruses. One study (87) compared antigen-capture enzyme immunoassay with cell cultures and determined that cell culture was more sensitive than antigen-capture and, in turn, that antigen-capture with polyconal antibody was more sensitive than with monoclonal antibody. A rapid monoclonal-antibody based antigen-capture test under a one-step strip format has been recently proposed (304). In a study using several procedures for detection of the virus in bursa of experimentally infected chickens, the RT-PCR was the most sensitive test (1, 4). Recent developments of the RT-PCR technique include multiplex RT-PCR (135) or real-time RT-PCR (171, 210) protocols aimed at detecting and differentiating the different strains of IBDV (classic, variant, and vvIBDV) directly from infected tissues. Provided an adequate dose effect curve is available, real-time RT-PCR may also be used to quantify the virus load in the studied sample (172, 210).

Differential Diagnosis

The sudden onset, morbidity, ruffled feathers, and droopy appearance of the birds in initial disease outbreaks are suggestive of an acute outbreak of coccidiosis. In some cases, there is blood in the droppings that would lead one to suspect coccidiosis. The muscular hemorrhages and enlarged edematous or hemorrhagic cloacal bursas would, however, suggest IBD.

Birds that die from IBD may show an acute nephrosis. Because of many other conditions that may cause nephrosis and the inconsistency of kidney lesions, such lesions should not be sufficient cause for a diagnosis of IBD. Again, involvement of the cloacal bursa usually will distinguish IBD from other nephrosis-causing conditions. Water deprivation will cause kidney

changes and possibly gray, atrophied bursas that closely resemble those associated with IBD infection. However, unless this occurs as a flock condition, such changes would be seen in relatively few birds. A history of the flock would be essential in aiding in the differential diagnosis of these cases.

Certain nephropathogenic strains of infectious bronchitis virus cause nephrosis (290). These cases can be differentiated from infectious bursal disease by the fact that there are no changes in the cloacal bursa, and deaths usually are preceded by respiratory signs. The possibility that the two diseases may occur simultaneously in a flock should not be overlooked.

The muscular hemorrhages and mucosal hemorrhages seen at the juncture of the proventriculus and gizzard are similar to those reported for hemorrhagic syndrome and could be differentiated on the basis of bursal changes that accompany IBDV infections. It is not unlikely that before IBD was recognized some cases were diagnosed as hemorrhagic syndrome.

Jakowski *et al.* (123) reported bursal atrophy in experimentally induced infection with 4 isolates of Marek's disease. The atrophy was observed 12 days post-inoculation, but the histologic response was distinctly different from that found in IBD (see Chapter 15).

Grimes and King (81) reported that experimental infections of 1-day-old, specific-pathogen-free (SPF) chickens with a type 8 avian adenovirus produced small bursas and atrophy of bursal follicles at 2 weeks post-infection. Several other organs such as the liver, spleen, pancreas, and kidneys were grossly affected, and intranuclear inclusion bodies were observed in the liver and pancreas.

Serology

The ELISA procedure is presently the most commonly used serological test for the evaluation of IBDV antibodies in poultry flocks. Marquardt *et al.* (163) first described an indirect ELISA for measuring antibodies, and since that time, several workers (25, 160, 247, 252, 262) have reported on the use of ELISA and its comparison to VN test results. The ELISA procedure has the advantage of being a rapid test with the results easily entered into computer software programs. With these programs, one can establish an antibody profile on breeder flocks that will indicate the flock immunity level and provide information for developing proper immunization programs for both breeder flocks and their progeny. To perform an antibody profile on a flock for the evaluation of the efficacy of vaccination programs, no less than 30 serum samples should be tested; many producers submit as many as 50–100 samples. The antibody profiles may be performed with serum collected either from the breeders or from 1-day-old progeny. If progeny serums are used, titers normally will be 60–80 percent lower than those in the breeders. It should be recognized that the indirect ELISA does not differentiate between antibodies to serotypes 1 and 2 (108) and that commercial kits may significantly detect antibodies to both serotypes (8). It should also be kept in mind that ELISA kits may also vary in sensitivity and specificity (50), and that being a very sensitive technique, ELISA may present both intra-laboratory and inter-laboratory variation (134). Thus, the introduction in the panel of tested sera of a reference sentinel serum with a known reactivity is advisable.

Prior to the use of the ELISA, the most common procedure for antibody detection was the constant virus-diluting serum VN test performed in a microtiter system (243). The VN test is the only serological test that will detect the different serotypes of IBDV and it is still the method of choice to discern antigenic variations between isolates of this virus. The indicator virus used for VN can make a significant difference in test results due to the fact that within a given serotype there are several antigenic subtypes (114). Significant discrepancies in the determination of virus neutralizing titers in different laboratories are also not uncommon (169). Most chicken serums from the field have high levels of neutralizing antibody to a broad spectrum of antigenically diverse viruses owing to a combination of field exposure, vaccine exposure, and cross-reactivity from high levels of antibody.

The other method used for the detection of IBDV antibodies is the AGP test. In the United Kingdom, a quantitative AGP test is routinely used (46); however, as used in the United States, the test is not quantitative. This test does not detect serotypic differences; it measures primarily antibodies to group-specific soluble antigens.

Treatment

No therapeutic or supportive treatment has been found to change the course of IBDV infection (42, 204). Because of the rapid recovery of the affected flock, treatments might appear highly effective if non-treated controls were not maintained for comparison. There are no reports in the literature concerning the use of some of the newer antiviral compounds and interferon inducers for the treatment of IBD.

Prevention and Control

The epidemiology of this infection has not been studied extensively, but it is known that contact with infected birds and contaminated fomites readily causes spread of the infection. The relative stability of this virus to many physical and chemical agents increases the likelihood that it will be carried over from one flock to a succeeding flock. The sanitary precautions that are applied to prevent the spread of most poultry infections must be rigorously used in the case of IBD. The possible involvement of other vectors (e.g., the lesser mealworm, mosquitos, dogs and rats) already has been discussed; they could certainly pose extra problems for the control of this infection.

Management Procedures

At one time, before the development of attenuated vaccine strains, intentional exposure of chicks to infection at an early age was used for controlling IBD. This could be advised on farms that had a history of the disease, and the chicks normally would have maternal antibodies for protection. Also, young chicks less than 2 weeks of age did not normally exhibit clinical signs of IBD. When the severe immunosuppressive effect of early IBD infections was discovered, the practice of controlled exposure with virulent strains became less appealing. On many farms, the

cleanup between broods is not thorough (including buildings and all materials and equipments), and due to the stable nature of the virus, it easily persists and provides an early exposure by natural means.

Immunization

Immunization of chickens is the principal method used for the control of IBD in chickens. Especially important is the immunization of breeder flocks so as to confer parental immunity to their progeny. Such maternal antibodies protect the chick from early immunosuppressive infections. Maternal antibody will normally protect chicks for 1–3 weeks, but by boosting the immunity in breeder flocks with oil-adjuvanted vaccines, passive immunity may be extended to 4 or 5 weeks (12, 148).

The major problem with active immunization of young maternally immune chicks is determining the proper time of vaccination. Of course, this varies with levels of maternal antibody, route of vaccination, and virulence of the vaccine virus. Environmental stresses and management may be factors to consider when developing a vaccination program that will be effective. Monitoring of antibody levels in a breeder flock or its progeny (flock profiling) can aid in determining the proper time to vaccinate. It should be mentioned that although they produce correlated antibody titers, the ELISA and VN tests may result in predicting different dates for vaccine susceptibility in progeny chicks (49). It is therefore advisable that the formulae used for calculating the dates of vaccination be extensively evaluated.

Many choices of live vaccines are available based on virulence and antigenic diversity. According to virulence, vaccines that are available in the United States are classed as mild, mild intermediate, intermediate, intermediate plus, or "hot." Vaccines that contain Delaware variants, either in combination with "classic" strains or alone, are also available. Highly virulent (hot), intermediate, and avirulent strains break through maternal VN antibody titers of 1:500, 1:250, and less than 1:100, respectively (148, 244). Intermediate strains vary in their virulence and can induce bursal atrophy and immunosuppression in 1-day-old and 3-week-old SPF chickens (152). If maternal VN antibody titers are less than 1:1000, chicks may be vaccinated by injection with avirulent strains of virus. Some vaccine virus replicates in the thymus, spleen, and cloacal bursa where it persists for 2 weeks (153). After the maternal antibody is catabolized, there is a primary antibody response to the persisting vaccine virus. A vaccine made by mixing an intermediate plus vaccine strain with a measured amount of IBDV antibody before injection has been used with some success to immunize day-old chicks in the presence of maternal antibody (82).

Killed-virus vaccines in oil adjuvant are used to boost and prolong immunity in breeder flocks. It has been proposed that their antigenic content may be measured by quantifying in the AC-ELISA the amount of VP2 or VP3 contained in the vaccine (156). Killed-virus vaccines are usually not practical or desirable for inducing a primary response in young chickens, however, injection of a fraction of a dose in broiler or pullet chicks between 1 and 10 days of age has sometimes been reported (296, 298). Oil-adjuvant vaccines are most effective in chickens that have been "primed" with live virus either in the form of vaccine (297) or field exposure to the virus. Oil-adjuvant vaccines presently may contain both standard and variant strains of IBDV. Antibody profiling of breeder flocks is advised to assess effectiveness of vaccination and persistence of antibody.

A more recent concept for the vaccination of chickens for IBD and other agents is *in ovo* vaccination at 18 days of incubation (73, 286). *In ovo* is a labor-saving technique and may provide a way for vaccines to circumvent the effects of maternal antibody and initiate a primary immune response. The injected material is a live IBD vaccine, either alone (73) or in combination with an anti-IBDV antibody so as to form immune complexes (286). The working mechanism of *in ovo* vaccination is not yet fully elucidated: *In ovo* injection of an intermediate IBD vaccine alone experimentally resulted in a faster recovery of bursal lesions, as compared with post-hatch vaccination, and in similar protection against challenge (215). Jeurissen proposed the working mechanism of the immune complex vaccine to be related to its specific cellular interactions with follicular dendritic cells in spleen and bursa (124). *In ovo* and post-hatch vaccinations have been recently reviewed by Negash *et al.* (190).

Advances in biotechnologies also allowed the development of new generations of vaccines, most of which are still experimental.

Production of IBD subunit vaccines has been attempted mainly from baculovirus- (164, 251, 273, 274, 302) or yeast- (158, 213) expressed proteins, with a report of expression using a Semliki forest virus vector (211). Several studies reported that baculovirus-expressed recombinant VP2 confered good to very good protection against IBDV challenge (164, 212, 273). The efficacy of baculovirus-expressed recombinant vaccines was shown to critically depend on the conformation and assembly of the expressed proteins (164), which process is now better understood (34, 35). A baculovirus-expressed VP2 protein has been used commercially in broiler breeders, and immunity was transferred to their progeny (302). More recently, Pitcovski *et al.* (213) described an oil-adjuvanted vaccine based on a purified recombinant VP2 antigen expressed in the *Pichia pastoris* yeast. This vaccine induced a protection level similar to conventional inactivated IBD vaccine and has been used in broilers in the field in Israel (213).

DNA vaccination is another approach, still experimental, based on plasmids expressing either the polyprotein gene (71, 31) or the VP2 gene alone (132, 283). Significant levels of protection were observed provided high amounts of plasmid DNA were repeatedly injected. Attempts have been made to increase the efficacy of IBD DNA vaccines, by co-administrating interleukine genes (104, 255) or synthetic oligodeoxynucleotides containing unmethylated CpG dinucleotide motifs (283) with an adjuvant effect, or by changing the route of administration of the DNA vaccine (88).

Finally, live recombinant virus vectors expressing IBDV immunogens have been reported. These include fowlpox virus (13, 28, 90), herpes virus of turkey (HVT) (48, 269), Marek's disease virus (268), CELO virus (72) and Newcastle disease virus (101). Additional recombinant vaccine viruses include IBDV vaccines that have been modified to broaden their antigenic spectrum

(183) or to allow the differentiation of vaccine induced antibodies (21). The only recombinant vaccine that has been licensed so far is derived from HVT and induces an active anti-IBDV antibody response facing high levels of neutralizing maternally derived antibodies (79).

A universal vaccination program cannot be offered because of the variability in maternal immunity, management, and operational conditions that exists. If very high levels of maternal antibody are achieved and the field challenge is reduced, then vaccination of broilers may not be needed. Vaccination timing with attenuated and intermediate vaccines varies from as early as 7 days to 2 or 3 weeks. If broilers are vaccinated at 1 day of age, the IBDV vaccine can be given by injection along with Marek's disease vaccine. Priming of breeder replacement chickens may be necessary, and many producers vaccinate with a live vaccine at 10–14 weeks of age. Killed oil-adjuvant vaccines commonly are administered at 16–18 weeks. Revaccination of breeders may be required if antibody profiling should indicate a major drop in flock titers.

The use of restriction fragment length polymorphisms of the VP2 gene of IBDV can be a powerful tool from an epidemiologic point of view. The original work by Jackwood and Sommer (120) described 5 molecular groups from 13 vaccine viruses and 5 IBDV isolates from the United States. When the same workers (121) examined 81 strains from around the world, they identified 16 more molecular groups. This certainly should indicate that this technique will identify strains of virus but does not indicate antigenic differences in the virus and, therefore, would not be helpful in predicting the immunogenicity of a vaccine.

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Chapter 8

Chicken Infectious Anemia Virus and Other Circovirus Infections

Introduction

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Classification

Circoviridae form a newly recognized family of viruses infecting mammalian and avian species (26). This family is related to a group of plant viruses known as nanoviruses (29). Porcine circovirus (PCV) was first described in 1974 as a picornavirus-like contaminant in PK15, a pig kidney cell line, without being linked to disease (23). This agent was named circovirus when it was shown that the genome consisted of circular, covalently linked single-stranded DNA. PCV had no similarities to any other known animal virus, but similar physicochemical properties were found in plant pathogens belonging to the geminiviruses (22). A second serotype of PCV (PCV2) has been described that is causing postweaning multisystemic wasting syndrome and perhaps other disease syndromes in piglets (reviewed in 1).

Viruses with similar physicochemical characteristics were detected in chickens and several psittacine species during the 1980s (20, 27). Notable viruses include psittacine beak and feather disease virus (PBFDV or BFDV) associated with feather dystrophy and loss, malformations of the beak and immunosuppression (16), chicken infectious anemia virus (CIAV) (31) and the recently recognized pigeon circovirus (PiCV) (30). These viruses as well as other avian circoviruses are described in detail in chapters 8.1 and 8.2.

Although these animal circoviruses have similar characteristics, there are major differences between CIAV on the one hand, and BFDV and PCV on the other hand. Based on the lack of DNA sequence similarities or common antigenic epitopes, replication strategies, and morphological differences, it was suggested that CIAV belongs to a separate virus group (12). Subsequent studies confirmed that the two groups are indeed different and that PCV, BFDV, and plant circoviruses are closely related (2, 11). The plant circoviruses are currently placed in the genus nanoviruses (9, 29). The vertebrate circoviruses may have evolved as the consequence of a recombinational event in which the N-terminus of the *rep* (replication initiator protein) gene came from a nanovirus and the C-terminal region came from a picorna-like virus (7).

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The taxonomy of the *Circoviridae* was changed in 1999 during the XIth International Congress of Virology in Sidney, Australia to better reflect the differences between the different members of the *Circoviridae*. CIAV was assigned as the only member to a new genus, *Gyrovirus*, while PCV and BFDV remained as the only recognized members of the genus *Circovirus* (14). Since then, canary circovirus and goose circovirus were also recognized as species in the genus *Circovirus*, while duck circovirus, finch circovirus, and gull circovirus are listed as tentative species (24).

Viruses with a similar genomic structure but with very limited sequence similarity to CIAV have been described in humans: the so-called TT viruses (TTV) and mini-TT viruses (TTMV). TTV was originally named after the initials of a patient in Japan, in whom it was first identified (reviewed in 3). The working group on the nomenclature of circoviruses has renamed these viruses as torquetenovirus and torquetenominivirus, respectively. These viruses are currently placed in a new genus *Anellovirus*, which is a floating genus unattached to an existing virus family (4). Since the identification of TTV, many similar viruses have been detected in nonhuman primates and other animals, but the clinical relevance of these viruses remains unclear (4).

Avian Circovirus or Circovirus-like Infections

Circoviruses or circovirus-like agents that are distinct from PBFDV, CIAV, and PiCV have been described in other avian species, both free-ranging and domestic. Companion and free-ranging avian species in which circovirus infections have been described include canaries (13), finches (10), starlings (8), ravens (19), a jay (Woods, personal observation), doves (15), and a gull (28). Domestic/poultry avian species with circovirus infections include geese (17), ducks (18), pheasants (21), and ostriches (5). Most infections have been associated with putative immunosuppression with or without feather abnormalities. The primary histopathological changes were lymphocytic depletion in the primary lymphoid tissues in addition to changes associated with secondary infections. In most of these reports, circoviruses or circovirus-like agents have been identified by electron microscopic or molecular-based methods (PCR, *in situ* hybridization).

Antibodies against CIAV were found in Japanese quail (*Coturnix coturnix japonica*) (6). It is not known whether the presence of the antibodies reflects infection of Japanese quail with CIAV or with a related virus. Todd *et al.* (25) suggested that additional circoviruses will probably be discovered in the future—most likely as one of the components in multifactorial diseases.

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Chicken Infectious Anemia

K. A. Schat and V. L. van Santen

Introduction

Chicken infectious anemia (CIA) was first recognized by Yuasa *et al.* (260) as a new disease in young chickens caused by a novel virus agent. The disease is characterized by aplastic anemia and generalized lymphoid atrophy with concomitant immunosuppression and is frequently complicated by secondary viral, bacterial, or fungal infections. The virus appears to play a major role in the etiology of a number of multifactorial diseases associated with hemorrhagic syndrome and/or aplastic anemia. Since the first description of this disease and subsequent isolation in cell culture of the virus in Japan (247), the virus has been isolated in virtually all countries with a poultry industry. In addition to anemia and associated syndromes, subclinical chicken infectious anemia virus (CIAV) infections, without anemia and increased mortality, are frequently observed in commercial flocks.

Definition and Synonyms

The terminology for the causative agent has varied over the years. The agent was originally designated chicken anemia agent (CAA) (260), but after morphologic and biochemical characterization (53, 123, 207), it was renamed chicken anemia virus (CAV) (53, 138). The name CAV has been accepted by the International Committee for the Taxonomy of Viruses (203). However, because the disease is commonly referred to as chicken infectious anemia, the causative virus is more logically referred to as chicken infectious anemia virus (CIAV) (239). This terminology will be used in this chapter.

CIA and closely associated syndromes have commonly been termed *hemorrhagic syndrome* (255), *anemia-dermatitis* (228), or *blue wing disease* (8, 48).

Economic Significance

Infection with CIAV has been confirmed as the cause of disease in chicken flocks between 2 and 4 weeks of age with syndromes suggestive of infectious anemia (12, 23, 28, 45, 63, 74, 101, 122, 162, 174, 192, 228, 241, 255). In these flocks growth was retarded and mortality was generally between 10 and 20%, but occasionally it reached 60%. In chickens 6 or more weeks of age, the etiologic significance of CIAV infection associated with aplastic anemia-hemorrhagic syndromes (64, 151, 257) has not definitely been established.

Infection with CIAV constitutes a serious economic threat, especially to the broiler industry and the producers of specific-pathogen-free (SPF) eggs. McIlroy *et al*. (112) reported a loss of net income of about 18.5% due to decreased weight at processing and increased mortality around 3 weeks of age in 15 broiler flocks. Approximately 29% of these broilers were derived from a common breeder flock, which was free of CIAV antibodies at 20 weeks of age, making the offspring susceptible to CIAV infection at a very early age. Interestingly, feed conversion ratios were not affected in the broilers with CIA. Davidson *et al.* (35) documented 14–24% reductions in weight of meat sold, as well as changes in feed conversion ratios, from CIAV-infected flocks exhibiting clinical signs characteristic of CIA.

Studies addressing the impact of subclinical CIAV infection on broiler flock performance have yielded conflicting results. In one study subclinically infected flocks in Northern Ireland yielded a 13% lower net income than CIAV antibody-negative flocks, mostly due to decreased weight at processing and suboptimal feed conversion ratios (125). A Belgian study demonstrated a higher slaughterhouse condemnation rate in CIAV positive flocks compared to CIAV negative flocks, but was unable to detect differences in other performance criteria (38). However, others were unable to confirm the negative influence of subclinical infection on production in the United States (61) and Denmark (96). In a retrospective case-control study in the United States, although presence of CIAV was found to be a risk factor for disease (gangrenous dermatitis, coccidiosis or respiratory disease), and disease was associated with production losses, the detection of CIAV alone was not associated with statistically significant decreases in performance or losses in production (69). It is likely, however, that the impact of subclinical infection with CIAV is underestimated, especially because subclinical infection reduces the development of antigen-specific cytotoxic T lymphocytes (CTL) significantly (109) and also adversely affects macrophage function (110).

The economic importance for the SPF industry is difficult to estimate, but seroconversion frequently occurs during the laying period (55, 246). As a consequence of seroconversion the flock is considered positive and the eggs are no longer SPF. The importance of this depends on legislation for vaccine production. The European Union requires the absence of CIAV from eggs used for the production of all poultry vaccines for administration in birds less than 7 days of age. Eggs from CIAV-positive flocks may be used according to USDA guidelines. Vaccines for human use, such as measles and mumps, require the use of eggs free of CIAV in Australia, Europe, and the United States.

Public Health Significance

CIAV infection has only been recognized in chickens, turkeys (177) and perhaps Japanese quail (50) and some European

Corvids (18), but circoviruses or circo-like viruses have been found in other species of birds and mammals (202). Results of serologic tests suggest that CIAV has no public health significance (239).

History

CIAV (Gifu-1 strain) was first isolated in 1979 in Japan by Yuasa *et al*. (260). However, the virus was present in chickens at least as early as 1970, when Jakowski *et al*. (87) described a condition of hematopoietic destruction in chickens with Marek's disease. The ConnB isolate of CIAV was later isolated from an ampoule of tumor cells obtained from these chickens (188, 243). The recent demonstration of CIAV antibodies in archived sera indicates that CIAV was present in chickens in the United States as early as 1959 (215).

A major breakthrough was achieved in 1983 when Yuasa *et al.* (257) reported that virus could be propagated in certain chicken lymphoblastoid cell lines, e.g., Marek's disease chicken cell (MDCC)-MSB1 (MSB1), causing cytopathic effects (CPE). This enabled the development of *in vitro* serological assays such as indirect immunofluorescence assays (234, 254) and virus-neutralization (VN) tests (234, 258). In addition virus could be easily purified from supernatant fluids of CIAV-infected cell cultures and characterized (53, 65, 80, 123, 207).

Virus identification was followed by studies that unraveled much of the pathogenesis and epizootiology of the infection. In the early 1990s, remarkable progress was made in research on the molecular biology of CIAV (reviewed in 144, 202). This resulted in the development of refined diagnostic methods and the potential for development of new types of vaccines (85, 99, 141).

Aplastic anemia syndromes, including inclusion-body hepatitis, were described many years before CIAV was detected. Their possible etiologic association with CIAV infection has been reviewed and discussed in several papers dealing with CIA (117, 166, 232).

Etiology

Classification

CIAV is classified as the only member of the genus *Gyrovirus* of the *Circoviridae* (167). The nomenclature of the *Circoviridae* is discussed in more detail in the introduction of Chapter 8.

Morphology

CIAV virions consist of nonenveloped, icosahedral particles with an average diameter of 25 to 26.5 nm using preparations negatively stained with 1% uranyl acetate (53, 80, 123). In such preparations, two types of virus particles differing in their orientation on the grid are commonly detected. Type I particles exhibit three-fold rotational symmetry and show a pattern of one central hollow surrounded by six neighboring hollows with a center-to-center distance of 7.5 nm, forming a regular surface network (Fig. 8.1B). Type II particles exhibit five-fold rotational symmetry and are characterized by 10 evenly spaced surface protrusions giving the impression of a cog-wheel structure (Fig 8.1A). The appearance of these particles suggested a regular T = 3 icosahedron with 32 morphologic subunits (53, 123). However, more recent modeling of unstained cryopreserved CIAV particles indicated a T = 1 lattice with 60 copies of VP1 in a capsid consisting of 12 pentagonal trumpet-shaped capsomeres. These protruding capsomeres distinguish CIAV from other *Circoviridae*, which have a smoother capsid surface (30).

Thin sections of CIAV-infected MSB1 cells, labeled with CIAV-specific monoclonal antibodies (MAb) and gold-labeled secondary antibodies, demonstrated the presence of intranuclear inclusions, often with a doughnut shape (123). All three viral proteins can be associated with apoptotic bodies, which show as electron-dense structures (42, 140, 210). In a minority of cells, virus particles were detected in the cytoplasm in association with microtubules (123).

Virions have a buoyant density in cesium chloride gradients variously reported as 1.33–1.34 g/mL (5, 207) or between 1.35 and 1.37 g/mL (53, 65). The sedimentation coefficient of CIAV has an estimated value of 91S in isokinetic sucrose gradients (5).

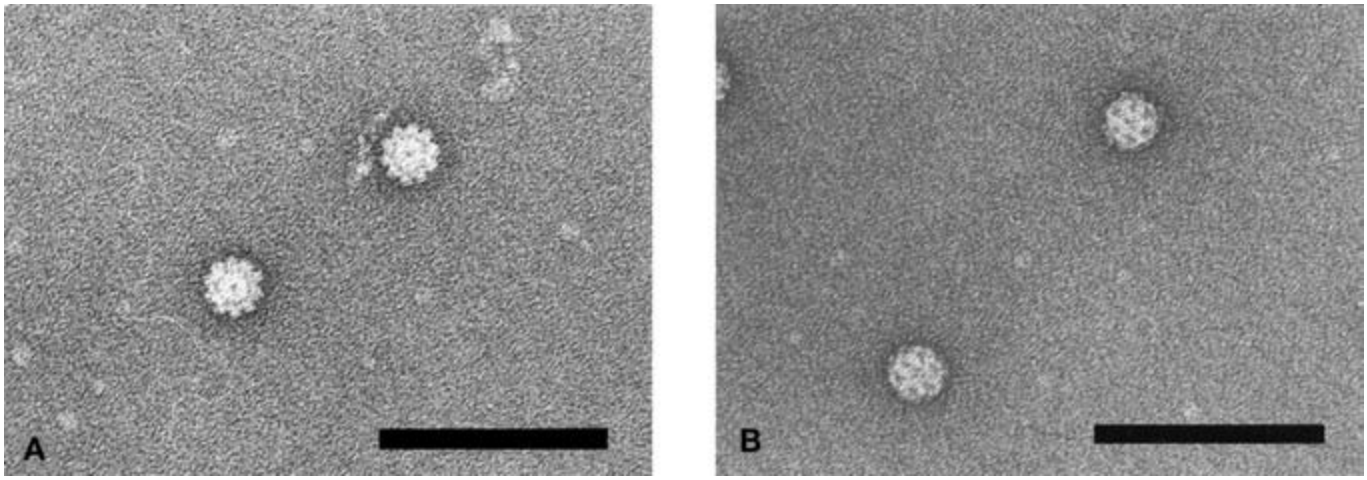
Chemical Composition

Viral DNA

The genome of CIAV consists of single-stranded, circular, covalently closed DNA (53, 207) of negative sense (138). The sequences of the complete genomes of many isolates have been determined (13, 24, 25, 51, 70, 84, 98, 127, 138, 170, 187, 224, 225, 244, 245). Nearly all CIAV viral genomes are 2298 nucleotides in length, and contain four 21-base direct repeats (DR), with a 12-base insert between the second and third DR. Todd *et al*. (204) reported that a fifth DR was obtained after about 30 passages of the Cux-1 strain in MSB1 cells, yielding a 2319-nucleotide genome. If the fifth repeat is present it is located upstream of the 12-bp insert. In infected cells, both single-stranded and double-stranded DNA are present, but virions contain only the circular minus-strand DNA (138, 161).

All sequenced strains have three partially overlapping ORFs coding for proteins of 52 (VP1, ORF1), 24 (VP2, ORF2) and 13 kDa (VP3, ORF3), one promoter region, and one polyadenylation signal. ORF3 is located within ORF2, and ORF2 partly overlaps ORF1. This genomic organization distinguishes CIAV from other members of the *Circoviridae*, which transcribe mRNA from both strands of their replicative intermediates, making their genomes ambisense (135).

The promoter-enhancer region, consisting of the four or five 21-base DR and the 12-base insert, is located upstream of ORF2 (143). The repeat units and the 12 bp insert contain recognition sites for different transcription factors (127, 131, 138). Optimal transcription requires both the DR and the 12 bp insert. The presence of the extra repeat enhanced transcriptional activity (143), whereas deletion of the first two DR reduced transcriptional activity by 40 to 50% (161). Disruption of the relative spacing of the DR region with other promoter elements and the start of transcription by insertion of a 7-bp linker decreases rate of virus replication in culture (142). The DRs contain sequences similar to the estrogen response element consensus half sites and compete with estrogen response elements for estrogen receptor bind-



8.1. Electron micrographs of chicken infectious anemia virus (CIAV). Different structural aspects of the CIAV capsids become apparent in negative-stained preparations. Two types of particle projections are obvious. A. Particle projection type II characterized by 10 peripheral protrusions. ×250,000. Bar = 100 nm. (Gelderblom) B. Projection type I showing CIAV capsids that exhibit 6 stain-filled morphologic units that surround one central hole.

ing in nuclear extracts (131). Expression from the CIAV promoter is higher in cells expressing estrogen receptor and is further increased by addition of estrogen (131). Noteborn (unpublished data cited in 142) found that CIAV DNA containing three DR but lacking the 12 bp insert was not able to produce viable virus particles. Although the 12-bp insert binds the transcription factor SP1 (143), substitution of a different 12-bp sequence did not further impair replication of virus already impaired by insertion of a 7-bp linker between the DR region and the rest of the CIAV promoter (142). Some changes in the length of the 12 bp insert region resulted in decreased cytopathogenicity and rate of spread in culture, but the virus neutralizing epitope was still produced (142). Sequences downstream of the transcription start site negatively regulate transcription (131).

Initially, only one unspliced, polycistronic mRNA of 2.1 kb, containing all three ORFs, was identified. Use of internal AUG start codons is required for the synthesis of VP1 and VP2 (139, 161). More recently, minor spliced mRNAs were identified in addition to the major unspliced mRNA by both Northern blotting and RT-PCR (97). One of the spliced mRNAs encodes a protein that shares amino- and carboxy-terminal sequences with VP1, but lacks 197 amino acids (positions 132–328) of VP1's 449 amino acids. Other spliced mRNAs encode novel proteins with frame-shifts. However, protein products of the spliced mRNAs have not yet been demonstrated.

Viral Proteins

A 50-kDa viral protein (VP1) is the only protein detected in highly purified virus particles (207). The N-terminal 40 amino acids show a limited similarity to histone proteins, suggesting a DNA binding role perhaps within the virus capsid (25, 127). The non-structural 30 kDa VP2 probably acts as a scaffold protein during virion assembly, so that VP1 folds in the proper way (99, 141). The third viral protein, VP3 (16 kDa) is associated with nuclei in infected cells (22, 42, 140), but not with highly purified virus particles (14).

Studies using neutralizing MAb on Western blots suggested that the neutralizing epitope(s) are conformational in nature and may consist of VP1 and VP2 components (14). This hypothesis was supported by studies showing that VP1 and VP2 were present in the same nuclear structures in infected cells (42). In addition, neutralizing antibodies were induced after inoculation of chickens with insect cells containing both VP1 and VP2, but not with cells containing only VP1 or VP2 (99). Virus-neutralizing MAb reacted with baculovirus-produced VP1 only if VP2 was co-produced. However, virus-neutralizing MAb bind to the native VP1 in virus capsids, which contain no VP2, but not to denatured VP1, lending further support to the role of VP2 as a scaffolding protein (141).

VP2 and VP3 are nonstructural proteins. VP2 is a multifunctional protein. In addition to its putative role as a scaffold protein enabling VP1 to attain its proper conformation, VP2 has serine/threonine and tyrosine protein phosphatase activities (159). VP3, also named apoptin, is a strong inducer of apoptosis in chicken thymocytes and chicken lymphoblastoid cell lines (140).

Virus Replication

Virions probably enter the cell by conventional adsorption and penetration. Low levels of the 2.1-kb polycistronic viral RNA transcript can be demonstrated at 8 hours post infection of MSB1 cells, with maximum levels attained at 48 hours (139, 161). Initially, only one unspliced mRNA of 2.1 kb and a minor transcript of approximately 4 kb were identified (161). More recently, minor spliced mRNAs in addition to the major unspliced mRNA were identified by both Northern blotting and RT-PCR (97).

Viral DNA replication occurs via a double-stranded replicative form (RF), probably by the rolling-circle mechanism (209). The

initiation site for the DNA replication has not been identified, although Bassami *et al*. (7) reported the presence of a nonanucleotide motif that may be involved in the initiation of DNA replication. Transfection experiments with the cloned tandemly-repeated CIAV RF suggest that homologous recombination can occur (209). This process can lead to double-stranded circular molecules that are identical to RF. The double-stranded RF may lead to the presence of latent episomal DNA and be responsible for the presence of viral DNA in gonadal tissues as reported by Cardona *et al*. (20). Todd (202) suggested that VP1 may have a role in DNA replication based on the presence of 3 amino acid motifs associated with rolling-circle replication of DNA.

VP3 can be detected at 6 hours, while VP2 is present at 12 hours post infection. The capsid protein VP1 is not detectable until 30 hours post infection (42).

The protein phosphatase activity of VP2 is very important, but not absolutely required, for CIAV replication. CIAV with a mutation of the catalytic cysteine of VP2 that abolishes both serine/threonine and tyrosine phosphatase activities (159) exhibits impaired replication and cytopathogenicity in MSB1 cells, reaching titers 10,000-fold lower than wild-type virus (160). Surprisingly, another VP2 catalytic site mutation that increases the tyrosine phosphatase activity and reduces serine/threonine phosphatase activity by only 30% impairs viral replication to a similar degree as the mutation abolishing both phosphatase activities (160). Other VP2 mutations, expected to have only subtle or no effects on protein phosphatase activity, resulted in varying degrees of impairment of CIAV replication (158). Unfortunately, the phosphatase activity of these other VP2 mutants was not reported. In addition to impaired replication, the VP2 mutants apparently exhibit reduced cytopathogenicity, which must be assessed subjectively, in culture. Interestingly, one VP2 mutation results in reduced cytopathogenicity without affecting viral replication efficiency, which suggests that viral replication functions and cyopathogenicity can be separated. In contrast to wild type CIAV, in cells infected with VP2 mutants, VP3 is cytoplasmic rather than nuclear in location, suggesting a role for VP2 in VP3 trafficking and function. This is significant, because the nuclear location of VP3 correlates with its ability to cause apoptosis in transformed, but not primary cells, where VP3 remains cytoplasmic (see below).

VP3 is essential for the virus replication cycle (Noteborn, unpublished data quoted in 140). Truncated apoptin lacking the last 11 amino acids is unable to induce apoptosis in transfected MSB1 cells (140). Interestingly, VP3 was found to induce apoptosis in several malignant human lymphoblastoid cell lines (264) and human osteosarcoma cells (265), but not in normal human cells (31). This finding has been confirmed in more than 70 cell types tested and extended to growth-transformed cells and cells from cancer-prone individuals exposed to UV irradiation (reviewed in 149 and unpublished observations cited in 172), but recently VP3 has been shown to induce apoptosis in a few normal human cell lines (68). Animal experiments using adenovirus vectors expressing VP3 suggest that VP3 may be used to treat humans with cancer (163, 221).

The potential of VP3 as an anti-tumor agent has stimulated investigation into the mechanism whereby it induces apoptosis in transformed cells. In order to induce apoptosis, VP3 must be phosphorylated on a specific threonine residue near the carboxy terminus by a cellular kinase, resulting in a predominantly nuclear location of VP3 (172, 263). This specific phosphorylation of VP3 also occurs in CIAV-infected MSB1 cells (172). Nuclear location of VP3 is necessary but not sufficient for induction of apoptosis (34). VP3 functions as a stable, but non-covalent multimer or aggregate containing approximately 30–40 copies (103). Considerable evidence has accumulated suggesting that VP3 triggers apoptosis via the intrinsic mitochondrial death pathway. Thus, VP3 triggers loss of mitochondrial membrane potential and release of cytochrome c and apoptosis-inducing factor from mitochondria (15, 33, 107). The apoptosis-inducing factor released from the mitochondria in response to VP3 is relocated to the nucleus, where it colocalizes with VP3 (107). Furthermore Apaf-1, a component of the apoptosome assembled after release of cytochrome c from the mitochondria, is required for VP3-induced apoptosis (15), whereas FADD and caspase 8, important signaling components of the extrinsic death receptor signaling pathway, are not (33, 107). Release of mitochondrial components triggered by VP3 results in activation of the cellular caspases 3 and 7, downstream caspases of the apoptotic pathway that are essential for the execution of programmed cell death (15, 33). Caspase 3 contributes to VP3-induced apoptosis, but is not essential, likely because other downstream caspases can substitute for caspase 3 (15, 107). The only evidence inconsistent with VP3 inducing apoptosis via the mitochondrial pathway are reports that Bcl-2, which inhibits activation of the mitochondrial death pathway, enhanced rather than inhibited VP3-induced apoptosis (32, 264). However, others have shown an inhibition of VP3-induced apoptosis by Bcl family members Bcl-2 and Bcl-X_L, consistent with other evidence that VP3 triggers apoptosis via the mitochondrial pathway (15, 107). No explanation for the contradictory results was proposed.

Recent studies have provided insight into the mechanism whereby VP3 triggers the intrinsic mitochondrial death pathway. The process does not require p53 (265) or cellular RNA or protein synthesis (34). VP3 possesses functional nuclear localization and nuclear export signals (34, 71, 164, 165) and its capacity to shuttle between the nucleus and cytoplasm is essential for its ability to induce apoptosis (71). Phosphorylation of the specific threonine residue, which is necessary for predominantly nuclear location of VP3, inhibits one of the nuclear export signals (164). VP3 associates with predominantly cytoplasmic anaphase-promoting complex and recruits it to nuclear PML bodies, which are known to be involved in apoptosis (71). Association of VP3 with the APC1 subunit of anaphase-promoting complex, which functions in the mitotic checkpoint of the cell cycle, disrupts the complex and results in degradation of its components and G2/M cell cycle arrest (199). The transduction of the apoptotic signal from the nucleus to the mitochondria is thought to depend on Nur77, which is essential for induction of apoptosis by VP3 (107). VP3 associates with Nur77, resulting in movement of Nur77 from the nucleus to mitochondria. Movement of Nur77 from the nucleus to mitochondrial outer membranes, resulting in release of

cytochrome c, also occurs in response to various other apoptotic stimuli that trigger the intrinsic death pathway (104). As mentioned above, mutations in VP2 reduce the nuclear location of VP3 in CIAV-infected cells (158). However with the exception of demonstration of phosphorylated VP3 in CIAV-infected cells (172), studies on the mechanism of apoptosis induction by VP3 have all been conducted in the absence of VP2, so the influence of VP2 on VP3 induction of apoptosis has not been examined.

Whether VP3 has functions in the CIAV replication cycle in addition to induction of apoptosis is not known. However, VP3 multimers form non-sequence-specific complexes with double- and single-stranded DNA and with RNA, with preference for ends in double-stranded DNA (102). Association with VP3 induces bends in DNA. These observations suggest VP3 could affect gene expression or DNA replication.

The replication of CIAV in very young chickens occurs primarily in hemocytoblasts in the bone marrow and T cell precursors in the cortex of the thymus (reviewed in 1, 133). Replication of the virus in the cortex of the thymus results in cell death by apoptosis (91) caused by VP3. Virus replication has also been demonstrated in other organs, where it is often but not always associated with lymphocytes (185). In chickens infected at 3 or 6 weeks of age, CIAV replicates in the thymic cortex, but CIAV-positive cells are rare in the bone marrow (184).

Resistance to Chemical and Physical Agents

Chicken infectious anemia virus is extremely resistant to most treatments (117). Yuasa (249) and Yuasa *et al*. (260) examined the effectiveness of different treatments to inactivate CIAV. Treatment of virus in liver suspensions with 50% phenol for 5 minutes inactivated CIAV but treatment with 5% phenol for 2 hours at 37°C was ineffective. The virus was found to be resistant to treatment with 50% ethyl ether for 18 hours and chloroform for 15 minutes. Treatment of liver suspensions with 0.1 N NaOH for 2 hours at 37°C or 24 hours at 15°C inactivates CIAV incompletely. Treatment with 1% glutaraldehyde for 10 min at room temperature, 0.4% β-propiolactone 24 hours at 4°C, or 5% formaldehyde 24 hours at room temperature inactivates the virus completely. Commercial disinfectants based on invert soap, amphoteric soap, or orthodichlorobenzene are not effective against CIAV. Treatments with iodine or hypochlorite are effective, but require 2 hours at 37°C with final concentrations of 10% rather than the generally recommended concentrations of 2%. Formaldehyde or ethylene oxide fumigation for 24 hours does not inactivate CIAV completely. The virus is also resistant to acid treatment at pH 3 for 3 hours. Treatments with disinfectants with pH 2 are widely used by the SPF industry and are apparently effective in inactivating the virus (55, 246).

CIAV is also resistant to treatment with 90% acetone for 24 hours (198). As a consequence, acetone-fixed slides of CIAV-infected material may remain infectious and need to be sterilized prior to final disposal. CIAV is resistant to heating at 56°C or 70°C for 1 hour and at 80°C for 15 minutes (45, 64, 260). However, it is only partially resistant to heating at 80°C for 30 minutes, and is completely inactivated within 15 minutes at 100°C (64). Inactivation of CIAV in infected chicken byproducts requires a core temperature of 95°C for 35 minutes or 100°C for 10 minutes, whereas fermentation was ineffective (220).

Strain Classification

Antigenicity

No antigenic differences have been recognized among various Japanese, European, and American isolates of CIAV using polyclonal chicken antibodies (45, 231, 234, 251). As a consequence, it is generally accepted that all strains belong to one serotype (117, 144). However, based on differences in reaction patterns with MAb (124, 178, 186) and DNA sequence differences resulting in changes in the predicted protein folding patterns (170), it is expected that strains may differ in their antigenicity.

A second serotype of CIAV, represented by CIAV-7, has been proposed (190, 191). CIAV-7 has characteristics such as small size and resistance to heat, acid, and chloroform similar to CIAV (191), and produces similar clinical disease and gross and microscopic lesions (190). However, thymic and bone marrow lesions and anemia produced by CIAV-7 are much milder than those generated by CIAV. Furthermore, the lack of any antigenic cross reactivity using polyclonal chicken sera and lack of cross hybridization under low-stringency conditions suggest that CIAV-7 is a novel virus rather than a new serotype of CIAV. Some progeny from several breeder flocks in the eastern United States are protected from CIAV-7 challenge, suggesting that the novel virus is prevalent in this region.

Molecular Differences

Partial or complete genome sequences of numerous strains from different parts of the world have been determined and amino acid sequences predicted. In general, most strains are very similar. Todd *et al*. (213) assigned CIAV isolates to seven groups based on restriction-enzyme analysis of a polymerase chain reaction (PCR)-amplified 675-bp fragment coding for the N-terminal half of ORF3. It is not clear whether these groups differ biologically. Minor differences in predicted amino acid sequences have been noted, especially for the amino acids 139–151 of VP1 (hypervariable region), and also at the carboxy terminus of VP2 and VP3 (170). The predicted protein structure of VP1 is affected by the observed differences in the hypervariable region (170). Similar differences in this region of VP1 have been reported for isolates from Brazil (137, 182) and Australia (13). Only two different hypervariable regions were found among 14 CIAV sequences from commercial broilers in one state in the United States (224). Genomes encoding these two hypervariable regions could be distinguished by restriction-enzyme analysis of a PCR product (224). Changes in the hypervariable region as well as other locations in VP1 occur during passage in culture (24, 70, 178, 244). Chimeric constructs in which a fragment encoding the hypervariable region had been exchanged between the highly passaged Cux-1 and low-passage CIA-1 strains demonstrated that differences in the hypervariable region influence virus replication in MSB1 cells (170). However, low-passage field isolates with different hypervariable regions could not be differentiated based on their replication in cell culture (225). The importance of the hypervariable region for *in vivo* pathogenicity is not clear. Meehan

et al. (126) examined the pathogenicity of a number of chimeric viruses with multiple changes, including those in the hypervariable region, and concluded that changes in the hypervariable region did not contribute disproportionately to pathogenicity. However, studies with changes only in this region have not been reported, and the question of the importance of the hypervariable region for the pathogenicity remains unresolved.

Although more variability has been found as more CIAV sequences become available, variability remains minor. Several authors have divided CIAV into groups based on nucleotide or amino acid sequence relationships (84, 100, 182, 224). These analyses indicate that genetic relatedness of CIAV sequences does not correspond to geographic location. Phylogenetic patterns based on nucleotide sequence comparisons and predicted amino acid sequence comparisons differ from each other (224). Furthermore, apparent phylogenetic relationships may be influenced by adaptation to culture (24). For these reasons, the significance of groupings based on phylogenetic relationships of CIAV sequences is unclear.

Pathogenicity

Although it is generally accepted that strains isolated worldwide do not differ substantially in pathogenicity, very few studies have directly compared different strains under identical experimental conditions. Yuasa and Imai (251) compared 11 isolates, which were each passaged 12 times in MSB1 cells prior to inoculation. Minor differences in virulence were found when chicks were inoculated at 7 days but not at one day of age; inoculation at 14 days of age failed to induce anemia. Natesan *et al.* (134) compared the pathogenicity of four isolates in one-day-old chicks and detected no differences. Toro *et al*. (217) reported thymus and bone marrow lesions when 10-week-old broiler breeders were infected with strain 10343, but comparative studies with other isolates in these birds were not reported.

Attenuation

Attenuation of CIAV (Cux-1) has been reported after as few as 49 passages in MSB1 cells by von Bülow and Fuchs (233). Pathogenicity was further decreased but not completely lost during additional passages up to 100 passages. Goryo *et al.* and Yuasa (65, 247) found no decrease in pathogenicity of other CIAV isolates after 19–40 passages, and Tan and Tannock (194) did not detect attenuation even after 129 passages. However, Todd *et al*. (204) found that Cux-1 became substantially less pathogenic after 173 passages in MSB1 cells. Attenuation resulted in genetically diverse virus populations. Molecularly cloned isolates from attenuated virus were indeed less pathogenic than the original isolate, but attenuation may not be stable. One isolate reverted back to pathogenicity after 10 passages in young chicks. However, because all molecular clones of the revertant virus tested were attenuated, the molecular change(s) responsible for recovery of pathogenicity could not be identified (206).

Additional passages of Cux-1 to passage 320 (p320) in MSB1 cells led to further attenuation (205). Nine molecular clones of p310 of Cux-1 were further analyzed for antigenicity and pathogenicity. Most clones were attenuated substantially although not completely; the number of chickens developing anemia after infection with most clones varied from 0 to 31%, while one clone caused anemia in 67% of the birds, versus 50–83% with the low passage virus (178). The pathogenicity of a substantially attenuated p320 molecular clone (CI 34) and a highly attenuated molecular clone (CRI 18) derived from CIAV passaged 173 times in MSB1 cells, ten times in SPF chickens, then seven times in MSB1 cells was investigated further (113). Neither attenuated CIAV caused reduction in hematocrit values. Thymic atrophy was absent in chickens infected with CRI 18 and milder and of shorter duration in chickens infected with CI 34 compared to low passage Cux-1. Flow cytometry showed that although both attenuated clones caused depletion of both $CD4^+$ and $CD8^+$ T cells in the thymus, depletion was significantly less than caused by low passage Cux-1. With regard to T cell depletion of the thymus, CRI 18 is more highly attenuated than CI 34. It is not known whether attenuation of these clones is stable after passage in chickens.

Differences between clones were also noted in reactivity patterns with MAb. Attenuated p320 clones exhibited reduced reactivity with neutralizing MAb 2A9, which reacts with a conformational epitope (178). Selection of a pool of MAb 2A9-resistant CIAV from p320 resulted in substantially attenuated virus, which produced no anemia or thymic atrophy in some experiments, compared to up to 90% anemic chickens produced by low passage virus. Investigation of the molecular basis of attenuation using a chimeric virus approach mapped the change(s) responsible for attenuation to the portion of the genome encoding the amino terminus of VP1 and the carboxy portions of VP2 and VP3 (179). Further studies of the basis for attenuation of p320 clones using chimeric viruses and CIAV with a site-specific mutation at VP1 codon 89 showed that change of VP1 amino acid 89 from threonine to alanine was necessary, but not sufficient, for attenuation exhibited by p320 CIAV clones. Cux-1 containing the mutation in VP1 codon 89 in conjunction with other changes in VP1 found in CI 34 (codons 75, 125, 141, and 144) exhibited pronounced attenuation, causing neither anemia, pale bone marrow nor thymic atrophy (214). Three of these changes (at codons 75, 125, and 144) substitute amino acids with similar properties and have also been observed in field isolates (84, 182, 224).

Yamaguchi *et al.* (244) selected molecular clones with reduced pathogenicity from a non-attenuated 10th passage virus pool of a different CIAV isolate, AH9410. Three of eight clones tested showed reduced ability to cause mortality and reductions in hematocrit and weight gain. A single amino acid difference (histidine at VP1 position 394) distinguished molecular clones with reduced pathogenicity from highly pathogenic molecular clones (glutamine at VP1 position 394). Introduction of this single amino acid change into a highly pathogenic molecular clone of a different CIAV isolate that had been passaged 39 times in MSB1 cells resulted in CIAV with greatly reduced pathogenicity; infection caused no mortality and resulted in hematocrits and weight gain indistinguishable from uninfected chickens. Viral loads in the liver of chickens infected with the attenuated viruses were approximately 10-fold lower than in chickens infected with the highly pathogenic clones. Effects of the attenu-

ated viruses on the thymus and T cells and stability of the attenuation were not examined.

Chowdhury *et al.* (24) found, based on ability to cause anemia and thymic atrophy, that two Malaysian CIAV isolates were attenuated by 60 passages in MSB1 cells. One of the isolates was further attenuated by passage up to passage 123. However, pathogenicity of the passaged viruses was not directly compared to low passage virus in the same experiment. Sequence analysis of the p60 viruses showed multiple changes in the predicted amino acid sequences of VP1. Surprisingly, the two isolates were more similar to each other after 60 passages than before passage, suggesting that specific changes had been selected during passage. Stability of attenuation and which changes were responsible for attenuation were not examined. Changes in VP1 amino acid 89 or 394, found by Todd *et al.* and Yamaguchi *et al.*, respectively, to be crucial for attenuation during passage in cell culture, were not found in Chowdhury's study, showing that different changes occurring during passage of CIAV in culture may each result in attenuation.

Laboratory Host Systems

CIAV can be propagated and assayed in cell cultures, 1-day-old chicks, or in chicken embryos.

Cell Cultures

The use of cell cultures is the preferred method for virus isolation and propagation since Yuasa (247) reported that some lymphoblastoid T cell lines (e.g., MDCC-MSB1 and MDCC-JP2) and the B cell line LSCC-1104B1 are suitable for propagation and assay of CIAV. However, many other T cell and B cell lymphoblastoid cell lines, whether producers or nonproducers of the respective transforming viruses, are resistant to CIAV (17, 235, 247).

Until recently MSB1 cell cultures were preferred for *in vitro* cultivation, although sublines of MSB1 differ in their susceptibility to infection. Some strains of CIAV, e.g., CIA-1 (105) may not replicate at all in one subline of MSB1 (MSB1-L) and poorly in another subline of MSB1 (MSB1-S), whereas both sublines are susceptible to infection with Cux-1 (170, 171). Furthermore, sensitivity of susceptible MSB1 cells to CIAV is reduced after subculturing the cells as little as 8 weeks (17, 234). Currently, the MDCC-CU147 (CU147) cell line seems to be the best cell line for propagation of CIAV, including the CIA-1 strain (17). In comparative assays, infection of CU147 with Cux-1 resulted in the detection of VP3 at 3 days post infection, while MSB1 cells did not become positive until 5 days post infection. Moreover, the percentage of positive cells was significantly lower in MSB1 cells than in CU147 cells at that time. Furthermore, the sensitivity of CU147 cells to CIAV is not reduced by subculturing up to 82 days. CU147 cells are also more sensitive than MSB1 cells for regeneration of CIAV from CIAV genomes molecularly cloned directly from field specimens without previous isolation of the virus (225).

Virus titrations require subculturing of inoculated cells every 2–4 days until cells inoculated with the endpoint dilution of CIAV are destroyed (234, 247). Alternatively, endpoints can be determined by PCR (223) or immunofluorescence (17) assay.

Chickens

Inoculation of one-day-old chicks free of maternal antibodies can be used to isolate and propagate CIAV in instances where the clinical syndrome suggests that CIAV may be present, but *in vitro* virus isolation and/or PCR assays are negative. Positive chicks develop anemia and gross lesions in lymphoid tissues and bone marrow after 12–16 days (260). Mortality may occur between 12 and 28 days post-inoculation but usually remains low, rarely exceeding 30%. Neonatally (253) or embryonally bursectomized (105) chicks can be used to enhance the sensitivity of isolation, especially if samples with low titers are analyzed. Chicks with maternal anti-CIAV antibody are resistant to CIAV infection and cannot be used for isolation or propagation of CIAV (256).

Chicken Embryos

Propagation of CIAV in chicken embryos following yolk sac inoculation has been reported by von Bülow and Witt (240). Moderate virus yields were obtained after 14 days from all parts of the embryo, but not from yolk or chorioallantoic membrane. Lesions were not observed after inoculation with the Gifu-1 and Cux-1 strains of CIAV. Some strains, however, may cause significant embryo mortality between 16 and 20 days of incubation. The CL-1 strain caused up to 50% mortality, with embryos being small, hemorrhagic, and edematous (101). The Australian vaccine strain 3711 also caused up to 50% embryo mortality (194).

Pathobiology and Epidemiology

Incidence and Distribution

Serological data had suggested that CIAV is ubiquitous in all major chicken-producing countries of the world (reviewed in 117, 232, 239). This was confirmed by virus isolation from chickens in all continents (176).

Natural and Experimental Hosts

The chicken is the only known host for CIAV. All ages are susceptible to infection, but susceptibility to anemia rapidly decreases in immunologically intact chicks during the first 1–3 weeks of life (64, 173, 251, 259, 260), although some strains have been reported to cause a reduction in hematocrit values after experimental infection of 10-week-old broiler breeders (217). Chickens 3 weeks of age and older continue to be susceptible to effects of CIAV on immune function (109, 110, 168, 184, 217).

Antibodies to CIAV have been detected in Japanese quail but not in ducks, pigeons and crows in Japan, but information on the specific species was not provided (50). Fancy chicken breeds in The Netherlands were frequently positive for CIAV antibodies (39). A survey in Ireland found CIAV antibodies in jackdaws, rooks and rare avian breeds, but not in pigeons, pheasants or ducks (18). McNulty *et al.* (120) failed to detect antibodies in turkey and duck sera. Turkey poults inoculated at 1 day of age with high doses of the virus were resistant to infection and did not develop antibodies to CIAV (unpublished data cited in 117). However, a circovirus similar to CIAV, but with low pathogenicity in chickens, has been isolated from turkeys (177).

Transmission

CIAV spreads both horizontally and vertically. Horizontal transmission is very likely based on the presence of high concentrations of virus in the feces of chickens for 5–7 weeks after infection (72, 258). Horizontal infection by direct or indirect contact most likely occurs via the oral route, but infection via the respiratory route, as shown in chicks after intratracheal inoculation (173), may also be possible in the field. Virus shedding occurs through the feces and perhaps through feather follicle epithelium as recently suggested by Davidson and Skoda (36). CIAV spreads easily among chickens in a group only if they are immunosuppressed (259). In field flocks naturally exposed to CIAV, it commonly takes 2–4 weeks until most birds have seroconverted (120, 189, 231). Isolation may prevent early seroconversion; 70% of grandparent flocks that were imported into Sweden and kept in quarantine remained seronegative until 16 weeks of age (46).

It is important to differentiate vertical transmission in commercial flocks from that in SPF flocks that may experience sporadic outbreaks. In the former, vertical transmission of virus through the hatching egg is considered to be the most important means of dissemination (23, 46). Vertical transmission of virus occurs when antibody-negative hens become infected by horizontal infection or by semen from infected cocks (73). Egg transmission only occurred from 8–14 days after experimental infection of hens (72, 261). After the development of immune responses egg transmission of virus could not be demonstrated, even when birds were stressed by injections with betamethasone or exchanging hens in cages. Field observations indicate that vertical transmission can occur during a period of 3–9 weeks after exposure with peak transmission at 1–3 weeks. The duration of egg transmission depends on the rate of spread of infection and development of immunity to CIAV (8, 23, 48, 228). In contrast to earlier studies showing the absence of vertical transmission after the development of antibodies, more recent studies have demonstrated CIAV DNA in progeny of hens with high titers of neutralizing antibody. Detection of CIAV relied on highly sensitive nested PCR, and no disease or lesions were found in the progeny (11).

Seroconversion patterns in SPF flocks suggest a more complex situation than described for commercial birds. Seroconversion in SPF chickens has been reported for commercial and noncommercial SPF flocks and often occurs during the first laying cycle (19, 49, 55, 119, 120, 132, 246, 254). When CIAV was accidentally introduced in 3 genetically different SPF flocks maintained at Cornell University, seroconversion coincided with the development of sexual maturity even while birds were housed in a CIAV-infected environment (19). Not all genetic strains seroconverted for 100% during a 60-week-period while birds were kept in colony cages in an environment contaminated with CIAV. However, CIAV DNA could be detected in seronegative and seropositive birds by nested PCR assays of gonadal tissues and spleens, even in chickens that had been antibody positive for more than 40 weeks (19, 20).

Horizontal spreading in SPF flocks kept in cages may be less efficient than described for field flocks. Miller *et al.* (132) followed seroconversion in a flock of 90 chicks hatched from eggs supplied by a SPF producer. All birds were bled monthly and one bird seroconverted at 6 weeks of age; this bird was euthanized. The remainder of the flock remained seronegative until 16 or 20 weeks of age when 2 additional birds became antibody positive. In a horizontal transmission experiment, a rooster shedding virus through semen was placed in a cage flanked by cages with seronegative chickens, which remained virus-negative over at least a 2-month-period (21). Significant differences in seroconversion rates were noted among genetic strains (19), but even between different generations of the same genetic strains, seroconversion ranged from 4% to 95% (132). Examination of tissues from embryos obtained from hens positive for viral DNA in the gonads showed that the embryos can carry the viral DNA without signs of virus replication, thus continuing the transmission cycle (130). These data strongly support the suggestion, first made by McNulty (117) that CIAV can establish a latent infection (133).

Incubation Period

In experimental infections, anemia and distinct histologic lesions can first be detected at 8 days after parenteral inoculation of virus. Clinical signs generally develop after 10–14 days, and mortality begins at 12–14 days after inoculation (66, 196, 260). Clinical signs are delayed and milder after oral inoculation compared to intramuscular inoculation (194, 222).

Under field conditions, congenitally infected chicks show clinical signs and increased mortality beginning at 10–12 days of age, with a peak at 17–24 days (23, 48, 63, 94, 228). In heavily infected flocks, there can be a second peak of mortality at 30–34 days (48, 94), probably due to horizontal transmission.

Clinical Signs

The only specific sign of CIAV infection is anemia, with a peak at 14–16 days post inoculation (PI). Anemia is characterized by hematocrit values ranging from 6 to 27%. Affected birds are depressed and can become pale. Weight gain is depressed between 10 and 20 days after experimental infection. Affected birds may die between 12 and 28 days PI. If mortality does occur, it generally does not exceed 30%. Surviving chicks completely recover from depression and anemia by 20–28 days PI (64, 173, 195, 237, 260), although retarded recovery and increased mortality may be associated with secondary bacterial or viral infections. Secondary infections, causing more severe clinical signs, are frequently seen in field cases, but they may also occur inadvertently in experimental chicks (47, 63, 228, 237).

Hematology

In general, hematocrit values greater than 27% are considered normal, but values may vary between inbred lines of chickens (86). Normal values are lower in white leghorn chicks than in broilers and decrease in both types of birds with increasing age (56, 57, 60). Blood of severely affected chicks is more or less watery, the clotting time is increased, and the blood plasma is paler than normal. Hematocrit values begin to drop below 27% at 8–10 days after infection, are mostly in the range of 10–20% at 14–20 days, and may even drop to 6% in moribund birds. In convalescent chicks, hematocrit values increase after 16–21 days and re-

turn to normal (29–35%) by 28–32 days post infection (66, 78, 174, 196, 260).

Low hematocrit values in CIAV-infected chickens are due to a pancytopenia as a consequence of infection of hemocytoblasts as early as 3–4 days PI (1, 185, 196), resulting in markedly decreased numbers of erythrocytes, white blood cells, and thrombocytes. Anisocytosis has been noticed as early as 8 days PI. Juvenile forms of erythrocytes, granulocytes, and thrombocytes begin to appear in the peripheral blood by 16 days PI, and the incidence of immature erythrocytes may exceed 30% several days later. The blood picture in convalescent chicks returns to normal by 40 days (196).

Decreased clotting is most likely the direct consequence of thrombocytopenia and may lead to the hemorrhages associated with CIA. Coinfection with infectious bursal disease virus (IBDV) may aggravate the thrombocytopenia (166).

Morbidity and Mortality

The outcome of CIAV infection is influenced by a number of viral, host, and environmental factors. Uncomplicated infectious anemia, especially if caused by horizontal infection, may result in nothing more than slightly increased mortality and transient poor performance of affected flocks, and, therefore, it could even go unobserved in commercial settings. However, subclinical infections with CIAV can aggravate other diseases (see "Immunosuppression").

Morbidity and mortality are considerably enhanced if chicks are dually infected with CIAV and Marek's disease virus (MDV), reticuloendotheliosis virus (REV), or IBDV, probably due to virus-induced immunosuppression (26, 152, 173, 236, 237, 238, 259). Because lymphocytic depletion of the bursa often precedes lymphocytic depletion of the thymus associated with CIAV infection in commercial chickens (189, 218), immunosuppression by other viral agents, such as IBDV, likely plays an important role in the outcome of CIAV infection in commercial flocks. Certain strains of reovirus also can be immunosuppressive in chickens (47, 180), which may explain the enhanced pathogenicity of CIAV in the presence of reovirus as reported by Engström *et al*. (47). Dual infections between *Cryptosporidium baileyi* and CIAV can enhance CIA as well as Cryptosporidiosis under experimental conditions (76). Occasional outbreaks of disease due to concurrent infections have been reported in commercial flocks (40).

Pathology

Lesions associated with CIA may vary dependent on the route of infection, age of exposure, viral dose, and immune status of the host. Moreover, CIAV infection may often be involved in and complicated by other pathogens. The pathology will be described for uncomplicated infections mostly based on experimental infections, as part of the hemorrhagic-aplastic anemia syndrome, and as a complicating factor in other diseases.

Gross Lesions

Thymic atrophy (Fig. 8.2A), sometimes resulting in an almost complete absence of thymic lobes, is the most consistent lesion especially when chicks develop age resistance to anemia (64, 89, 184, 196). The thymic remnants may have a dark reddish color. Bone marrow atrophy is the most characteristic lesion seen and is best evaluated in the femur (66, 196). Affected bone marrows become fatty and yellowish or pink (Fig. 8.2B). In some instances, its color appears dark red, although distinct lesions can be detected by histologic examination. Bursal atrophy is less commonly associated with CIAV infection. In a small proportion of birds, the size of the bursa of Fabricius may be reduced. In many cases, the outer bursal wall appears translucent, so plicae become visible. Hemorrhages in the proventricular mucosa and subcutaneous and muscular hemorrhages are sometimes associated with severe anemia (64, 66, 105, 174, 195, 196, 237). More pronounced hemorrhages or bursal atrophy, and lesions in other tissues—e.g., swollen and mottled livers (66, 174)—have also been reported but may be caused by secondary infections with other agents.

Hemorrhagic-aplastic Anemia Syndrome

Outbreaks of infectious anemia in field flocks are mostly associated with the so-called hemorrhagic syndrome, with or without concurrent (gangrenous) dermatitis (Fig. 8.2C) (e.g., 8, 23, 41, 48, 228, 255). CIAV is also involved in the etiology of aplastic anemia associated with inclusion body hepatitis (IBH) (238) and with the IBH/hydropericardium syndrome (216) or infectious bursal disease (166). Hemorrhages seen in chickens with infectious bursal disease may, in most instances, be a consequence of CIAV rather than IBDV infection.

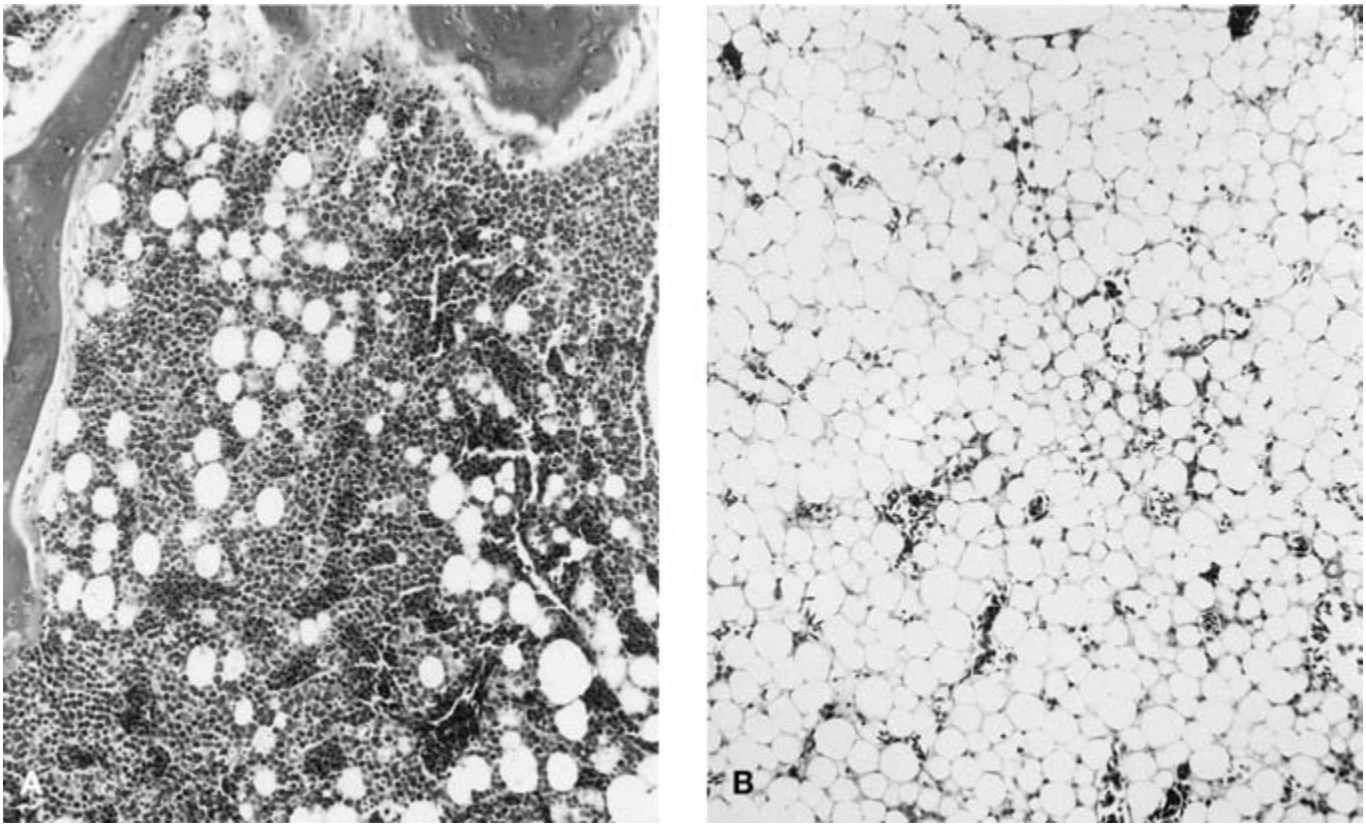
Characteristic lesions of so-called hemorrhagic syndrome are intracutaneous, subcutaneous, and intramuscular hemorrhages (Figs. 8.2D, E). Punctuate hemorrhages may be present even more frequently in the mucosa of the distal part of the proventriculus (Fig. 8.2F). Intracutaneous or subcutaneous hemorrhages of the wings are often complicated by severe edema and subsequent dermatitis, which may become gangrenous due to bacterial infection (48).

Subcutaneous hemorrhage of shanks and feet may result in formation of ulcers. Affected chicks also sometimes appear to be predisposed to develop pododermatitis.

Hemorrhages are not consistently seen in anemic chicks, although their occurrence is mostly correlated with the severity of anemia. Increased clotting time associated with thrombocytopenia, therefore, does not completely explain hemorrhages. Endothelial lesions and impaired liver functions, partly caused by viral infection and enhanced by secondary bacterial infection, are likely to be more important in the pathogenesis of hemorrhagic diathesis.

Microscopic Pathology

Histopathologic changes in anemic chicks have been characterized as panmyelophthisis and generalized lymphoid atrophy (66, 90, 105, 166, 185, 195, 196, 235, 237). In the bone marrow, atrophy and aplasia involve all compartments and hematopoietic lineages (Fig. 8.3). Necrosis of residual small cell foci may occasionally be seen. Hematopoietic cells are replaced by adipose tissue or proliferating stroma cells. Regenerative areas consisting of proerythroblasts appear 16–18 days after experimental infec-



8.3. Femoral bone marrow from 14-day-old chickens. A. Uninfected control. B. Chicken infectious anemia virus-infected, 14 days postinoculation. Note atrophy of hematopoietic tissue and presence of fat cells. H & E, ×160. (*Lucio and Shivaprasad*)

tion, and there is a hyperplasia of bone marrow between 24 and 32 days PI in birds that recover.

Severe lymphoid depletion is seen in the thymus, starting with the cortical lymphocytes, but the nonlymphoid leukocytes and stroma cells are not affected. The thymus cortex and medulla become equally atrophic, with hydropic degeneration of residual cells and occasional necrotic foci (Fig. 8.4). In chicks that recover, repopulation of the thymus with lymphocytes becomes distinct at 20–24 days, and the morphology returns to normal by 32–36 days PI.

Lesions in the bursa of Fabricius may be present. These lesions consist of mild to severe atrophy of the lymphoid follicles with occasional small necrotic foci, infolded epithelium, hydropic epithelial degeneration, and proliferation of reticular cells (Fig. 8.5). Repopulation of lymphocytes until complete recovery is similar to that in the thymus.

In the spleen, depletion of T cells with hyperplasia of reticular cells is seen in the lymphoid follicles as well as in the Schweigger-Seidl sheaths. Necrotic foci in follicles or sheaths have been observed rarely.

In the liver, kidneys, lungs, proventriculus, duodenum, and cecal tonsils, lymphoid foci are depleted of cells, making them smaller and less dense than those in unaffected birds. Liver cells are sometimes swollen, and hepatic sinusoids may be dilated.

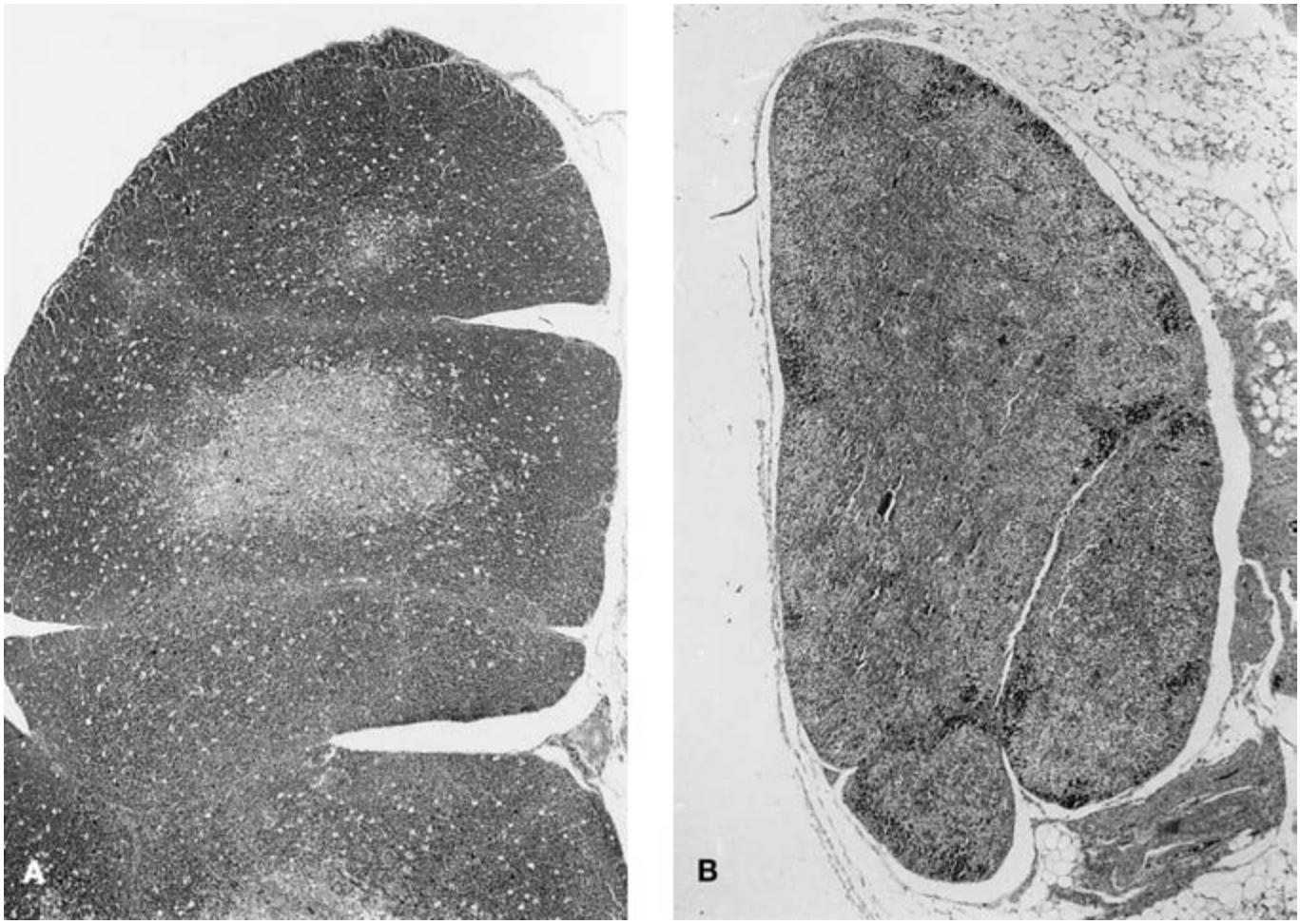
Small eosinophilic nuclear inclusions have been detected in altered, enlarged cells of affected tissues, predominantly in the thymus and bone marrow, where they are most frequent at 5–7 days after experimental infection (66, 185).

Ultrastructural Lesions

Few ultrastructural studies have been described for CIAV-infected chicks (62, 67, 91). Changes in hematopoietic cells and thymocytes were first observed at 6 days PI and were most advanced at 8 days PI. The affected cells had electron-dense regions in the cytoplasm and inclusion bodies consisting of homogeneous or fine granular materials. In addition, irregular plasma membranes, vacuolization, and pseudopod formation were seen. Between days 12 and 16 PI, many degenerative cells were seen, as well as actively phagocytizing cells. Apoptotic bodies were present in infected thymocytes. After 20 days, regeneration started to occur.

Pathogenesis

The basic events during the pathogenesis of CIAV infection have been elucidated by sequential histopathologic (66, 184, 185, 196), ultrastructural (62, 67, 91) and immunocytochemical studies (75, 184, 185). Hemocytoblasts in the bone marrow and lymphoblasts in the thymus cortex are primarily involved in early cytolytic infection at 6–8 days PI leading to a rapid depletion by apoptosis of these cells. Besides enlarged proerythroblasts and degenerating hematopoietic cells, macrophages with ingested degenerated hematopoietic cells have been observed in the bone



8.4. Thymus from 14-day-old chickens. A. Uninfected control. B. Chicken infectious anemia virus-infected, 14 days postinoculation. Note the absence of demarcation between medulla and cortex. H & E, ×63. (*Lucio and Shivaprasad*)

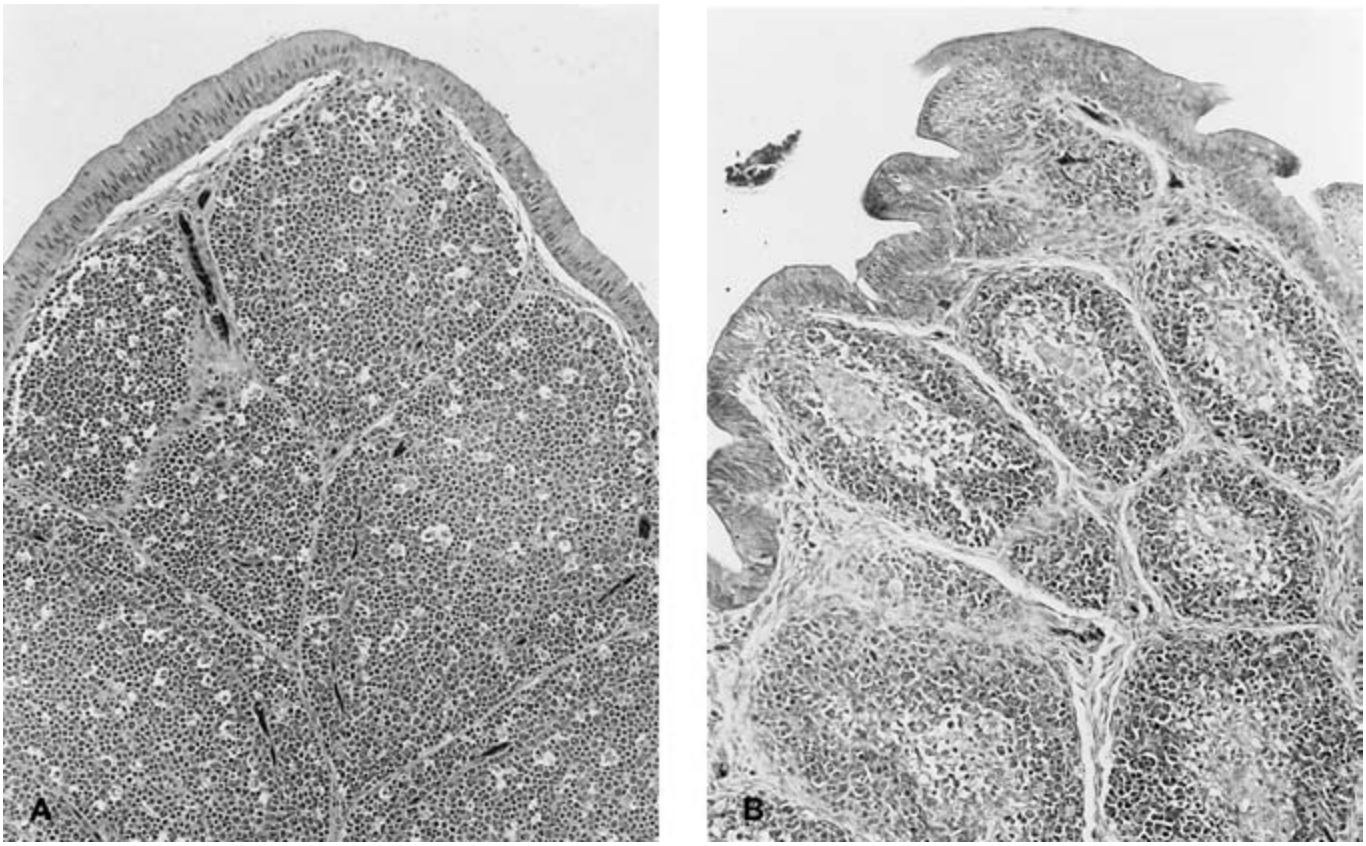
marrow. In contrast to the thymus, depletion of lymphoid cells and occasional necrosis in the bursa of Fabricius, spleen, and lymphoid foci of other tissues have not been detected before 10–12 days PI (66, 185, 196, 237). Repopulation of the thymus with lymphocytes, repopulation of the bone marrow with proerythroblasts and promyelocytes, and recovery of hematopoietic activity beginning 16 days PI all appear to coincide with the beginning of antibody formation (see "Immunity"). These events result in complete recovery by 32–36 days.

Treatment of formalin-fixed thymus tissues with proteases III or XIV, needed to unmask viral antigens (116), facilitated immunocytochemical studies (2, 75, 79, 185). Large numbers of cortical thymic lymphoblasts become virus-positive within 4 to 6 days PI. In addition, intrasinoidal and extrasinoidal hemocytoblasts, reticular cells in the bone marrow, and mature T cells in the spleen can be vira antigen-positive. Infected cells in the thymus and bone marrow are most abundant at 6–7 days PI and can be detected until 10–12 days or even later. Viral antigen has also been demonstrated in lymphoid tissues in many other organs (185). Infection of proventriculus, ascending part of the duodenum, kidney, and lung, could provide an explanation for virus shedding. Infected cells in these tissues usually cannot be detected for more than 22 days after infection at 1 day of age (185), although virus may persist in tissues until 28 days and in rectal contents until 49 days or later (258).

Although CIAV has a tropism for lymphoid tissue, particularly for the thymus cortex (90), susceptibility of thymocytes or spleen cells to infection is not dependent on the expression of particular cell markers such as CD4 or CD8 (2, 90). On the other hand, transient severe depletion of $CD4^+$ and $CD8^+$ lymphocytes, or a selective decrease in CTL, may play an important role in the mechanism of CIAV-induced immunosuppression (2, 26, 79, 90).

Age Resistance

Under experimental conditions, age resistance to anemia develops rapidly during the first week of life and becomes complete by 3 weeks or even earlier in immunologically competent chickens. The degree of resistance may vary based on the virulence of the virus, dose, and route of infection (64, 173, 174, 217, 251, 259). Development of age resistance is closely associated with the ability of the chicken to produce antibodies against the virus (251, 259). Chickens infected at 6 weeks of age with high doses of



8.5. Bursa of Fabricius from 14-day-old chickens. A. Uninfected control. B. Chicken infectious anemia virus-infected, 14 days postinoculation. Note lymphoid depletion and atrophy of follicles. H & E, ×63. (*Lucio and Shivaprasad*)

CIAV rapidly develop neutralizing antibodies and do not shed virus, whereas chickens infected with a lower dose require more time to develop detectable antibody and do shed virus (43). Antibody development is considerably delayed by immunosuppression, e.g., by simultaneous infection with infectious bursal disease virus (IBDV) (81, 173, 259) or by bursectomy (78, 253). Dual infection with IBDV increases the persistence of CIAV in blood cells of chickens infected at 6 weeks of age and prolongs viral shedding (81).

Although most CIAV experiments have been conducted in leghorn chickens, Joiner *et al.* (92) showed that commercial broilers also exhibit age resistance to clinical disease and have low viral loads under experimental conditions. However, under commercial conditions, anemia in association with CIAV infection and accompanied by bacterial and parasitic diseases indicative of immunosuppression is observed in chickens up to 130 days of age (35, 219, 242). This suggests that the concept of age resistance may not always be valid in commercial operations, possibly due to environmental factors or other pathogens that affect the immune function of the flocks.

Jeurissen *et al*. (89) suggested that age resistance was dependent on susceptibility of thymic precursor cells during prehatching and posthatching development. However, embryonally bursectomized chickens remained fully susceptible and developed thymus atrophy and anemia when challenged at 5 weeks of age (78). Moreover, mononuclear cell cultures established from thymus, spleen, and bone marrow tissues from 28-day-old chicks were fully susceptible to infection (115). Furthermore, CIAV replicated in the thymic cortex of chickens infected at 3 or 6 weeks of age, resulting in extensive depletion of the cortex, while CIAV positive cells were rare in the bone marrow and other tissues (184). Several studies have demonstrated lack of age resistance to the subclinical disease characterized by reduced cellular immune responses (109, 110, 168, 184, 217).

Route of Infection and Virus Dose

Virus dosage affects the severity of anemia or the proportion of affected chicks, but infection can be established by intramuscular injection of doses as low as $10^{0.75}$ 50% tissue-culture-infective-doses ($TCID_{50}$) (121, 173, 260). The route of infection also plays a role in experimental infection, because infection by contact usually does not cause anemia in immunologically intact chicks, in contrast to immunologically compromised birds (173, 259). Oral, nasal, or ocular infection routes are much less effective than parenteral inoculation in inducing disease (173, 194, 222, 248).

Genetic Resistance

There is little information on genetic resistance to infection and disease. Hu (77) suggested that S13 (MHC: $B^{13}B^{13}$) chicks seemed to be more susceptible to disease than N2a (MHC:

$B^{21}B^{21}$) and P2a (MHC: $B^{19}B^{19}$) chicks. This observation is compatible with the finding that S13 chickens have a poor seroconversion rate after natural exposure and after vaccination with a commercial vaccine using an adjuvant. S13 chickens had only a 73% seroconversion 7 weeks post vaccination, while the N2a and P2a strains were 100% and 85% seropositive, respectively (19). In an experiment designed to detect MHC influences on CIAV susceptibility in four-week-old broiler chickens, Joiner *et al.* (92) found no statistically significant differences among MHC types in seroconversion rates and viral loads 2 weeks PI.

Immunity

Active Immunity

Antibody responses are the major arm of protective immunity to CIAV, but neutralizing antibodies cannot be detected until 3 weeks PI of susceptible one-day-old chicks. Titers are low (1:80) and show little increase (1:320) until 4 weeks. Chickens inoculated intramuscularly at 2–6 weeks of age have a faster response with neutralizing antibody detectable as early as 4–7 days and with maximum titers (1:1280–1:5120) at 12–14 days PI (43, 254, 258). Humoral antibody formation is delayed if chickens are infected orally rather than intramuscularly (194, 222). Yuasa *et al*. (258) reported that increasing antibody production coincides with decreasing virus concentrations in chicken tissues. However, comparing the levels of antibody detectable by ELISA at 14 days PI among individual chickens inoculated at 4 weeks of age, Joiner *et al*. (92) found that higher virus levels corresponded to higher antibody levels, suggesting that higher antibody levels were a result of greater stimulation by virus.

Seroconversion in horizontally infected breeder flocks may be detected as early as 8–9 weeks of age, and most flocks have antibodies to CIAV at 18–24 weeks (82, 120). High titers of neutralizing antibody persist in all birds of a flock for at least 52 weeks. The prevalence of antibodies detected by indirect immunofluorescence assays, however, may decrease with increasing age (82) and is frequently less than 100% in a flock (58, 120). Antibodies detected by a commercial ELISA kit will remain present until 60 to 80 weeks of age in CIAV-infected SPF flocks (19). There is no information on the importance of cell-mediated and non-specific immunity, although Hu *et al*. (78) noted that some embryonally bursectomized birds recovered from anemia in the absence of antibodies.

Passive Immunity

Maternal antibodies provide complete protection of young chicks against CIAV-induced anemia (256). This protection can be abrogated if chicks are immunosuppressed by other factors, including viral infections, especially infections, such as IBDV, that affect humoral immune responses (173, 237). Maternally derived immunity, including protection against experimental challenge, persists for about 3 weeks (120, 154). Furthermore, vertical transmission of the virus is unlikely to occur from antibody-positive hens, but viral DNA can still be transmitted (11, 19, 20, 130). Outbreaks of infectious anemia in the field are in fact correlated with the absence of anti-CIAV antibody in the parent flocks (23, 46, 228, 255).

Immunosuppression

Impairment of the immune response by CIAV infection may result directly from damage to hematopoietic and lymphopoietic tissues and subsequent generalized lymphoid depletion or perhaps from cytokine imbalances. Splenocytes from experimentally infected 1 to 7-day-old chicks had depressed responses to mitogen stimulation between 7–15 days but not at 18–21 days PI (3, 9, 150, 155). Depressed mitogen responses were also noted between 14 and 21 days after oral infection of 3-week-old chickens (110). Decreases in macrophage functions such as Fc receptor expression, Interleukin (IL)-1 production, phagocytosis, and bactericidal activity were noted after infection of one-day-old and three-week-old chicks (110, 111). Although transient, effects on macrophage function persisted longer than effects on T-cell mitogen responses, up to 6 weeks PI (110, 111). Interferon (IFN) production by mitogen-stimulated splenic lymphocytes *in vitro* was increased at 8 days and decreased between 15 and 29 days PI (3, 4, 110). IFN-γ mRNA levels were also increased in the spleens of CIAV-infected chickens 7 days PI (109). Although IFN-γ mRNA levels in the spleen subsequently decreased, at 14 days PI they were not reduced compared to uninfected control chickens (109). T cell growth factor production (presumed to be IL-2) after *in vitro* stimulation was also decreased between 14 and 21 days PI (110), but a decrease in levels of IL-2 mRNA in spleens of infected chickens was not detected (109). CIAV infection interfered with the increase in both IFN-α and IFN-γ mRNA levels in blood cells induced within four hours in response to vaccination with trivalent inactivated IBDV/NDV/IBV vaccine (168). This marked effect on early innate responses was found 1, 2 and 3 weeks post CIAV infection of 4-week-old chickens. Markowski-Grimsrud and Schat (109) found that CIAV infection significantly reduced the development of antigen-specific CTL for MDV and REV in chickens infected with CIAV after 3 weeks of age, suggesting that CIAV can impact vaccinal immunity and recovery from infections when cell-mediated immune responses are important. There is apparently no "age resistance" to the immunosuppressive effects of CIAV.

CIAV as a Co-factor in Other Diseases

Based on the impact of clinical or subclinical CIAV infection on specific and non-specific immune responses, it is not surprising that infection has been linked to increased susceptibility to other pathogens. Immunosuppression in anemic CIAV-infected birds has been linked to increased bacterial and fungal infections (63, 169, 195, 235, 241) and to enhanced pathogenicity of adenovirus (216, 238), reovirus (47) and infectious bronchitis virus (IBV) (218, 226). van Santen *et al.* (226) found infection with CIAV delayed development of infectious bronchitis virus IBV-specific IgA in tears, prolonged respiratory signs, and delayed IBV clearance in IBV-infected chickens. Experimental infection with CIAV and *Salmonella enterica* Serovar Enteritidis resulted in a decrease in the number of gut-associated T cells and IgA^+ cells and the level of intestinal *Salmonella*-specific IgA compared to chickens infected with *Salmonella* alone. However, there was no significant increase of *Salmonella* positive cells in dually infected birds (181). Hagood *et al.* (69) found a significant associ-

ation between both presence of CAV DNA and thymic atrophy and coccidiosis, gangrenous dermatitis, or respiratory disease in commercial broiler flocks. De Boer *et al.* (37) used live attenuated Newcastle disease (ND) LaSota-type vaccine in 1- and 10-day-old chicks, which were infected at one day of age with CIAV. Severe respiratory distress was noted in the dually infected chicks without affecting the HI titers against ND virus. Impaired humoral immune response to inactivated ND vaccine may also occur (10, 27) but is not a usual phenomenon in commercial flocks (59).

Dual infections with CIAV and MDV have led to increased early mortality and increased incidence of MD (52, 151, 236, 262). A high proportion of MDV isolates from 14–24 week-old layers exhibiting acute MDV infection also contain CIAV, and inoculation of CIAV into commercial chickens reactivates latent MDV infections (52). Two factors may influence the degree of interactions. Infection with a low dose of MDV enhanced lymphoproliferative MD lesions, while high doses decreased the lesions (88). The virulence of the MDV strain may also influence the outcome of dual infections. Miles *et al*. (129) found that coinfection with CIAV and very virulent (vv)MDV strains exacerbated the mortality and thymus atrophy, but that this was less evident with vv+MDV strains. MDV vaccinal immunity is depressed by CIAV infection even if infection occurs at 14 days of age (150, 155, 252) and, based on the ablation of MDV-specific CTL responses, (109) probably at later times as well.

Diagnosis

Isolation and Identification of CIAV

Detailed procedures for the isolation and identification of CIAV have been published (118). Virus can be isolated from most tissues, buffy coat cells, and rectal contents from diseased chickens with maximum virus titers detected at 7 days after infection (118, 258). Virus titers will decrease after antibodies develop, but whole blood, buffy coat cells, and thymic homogenates were found to be infectious for at least 14 days PI, even in birds with neutralizing antibody (222, 236, 250).

Liver or lymphocytes from the spleen or buffy coat are preferred sources for virus isolation. Clarified homogenates can be heated for 5 minutes at 70°C (64) or treated with chloroform to eliminate or inactivate possible contaminants before inoculating cell cultures.

MDCC-CU147 or MSB1 cell cultures are preferred for virus isolation and titrations (17, 247). Some CIAV strains do not readily replicate in MSB1 and differences in susceptibility of MSB1 sublines have been reported (170). Freshly prepared cultures containing 2×10^5 cells/mL and seeded at 10^5 cells/cm^2 should be used. Cells are inoculated with 0.1 mL/1 mL of culture with 1:20 or greater dilutions (or serial 10-fold dilutions) of appropriately prepared tissue homogenates. Cultures are split every 2–4 days for 10 passages or until cell death is observed. Microscopic examination of cultures between 36 and 48 hours after passage is recommended to distinguish between virus-induced cytopathic effects (Fig. 8.6) and nonspecific cell degeneration. Isolation of CIAV should be verified by PCR analysis.

Bioassay by intramuscular or intraperitoneal inoculation of susceptible 1-day-old chicks is the most specific method available for primary isolation of CIAV. This approach can be used if CIAV is suspected, but virus cannot be isolated in cell culture. The bioassay is as much as 100-fold more sensitive than cell culture and the sensitivity can be further increased by bursectomy (78, 253). Between 14 and 21 days after inoculation hematocrit values are examined; values below 27% are considered indicative of the presence of CIAV (105, 174). Postmortem examination for bone marrow atrophy can be used in the case of nonanemic birds. Confirmation that CIAV is present in the lesions by PCR or immunohistochemistry is important.

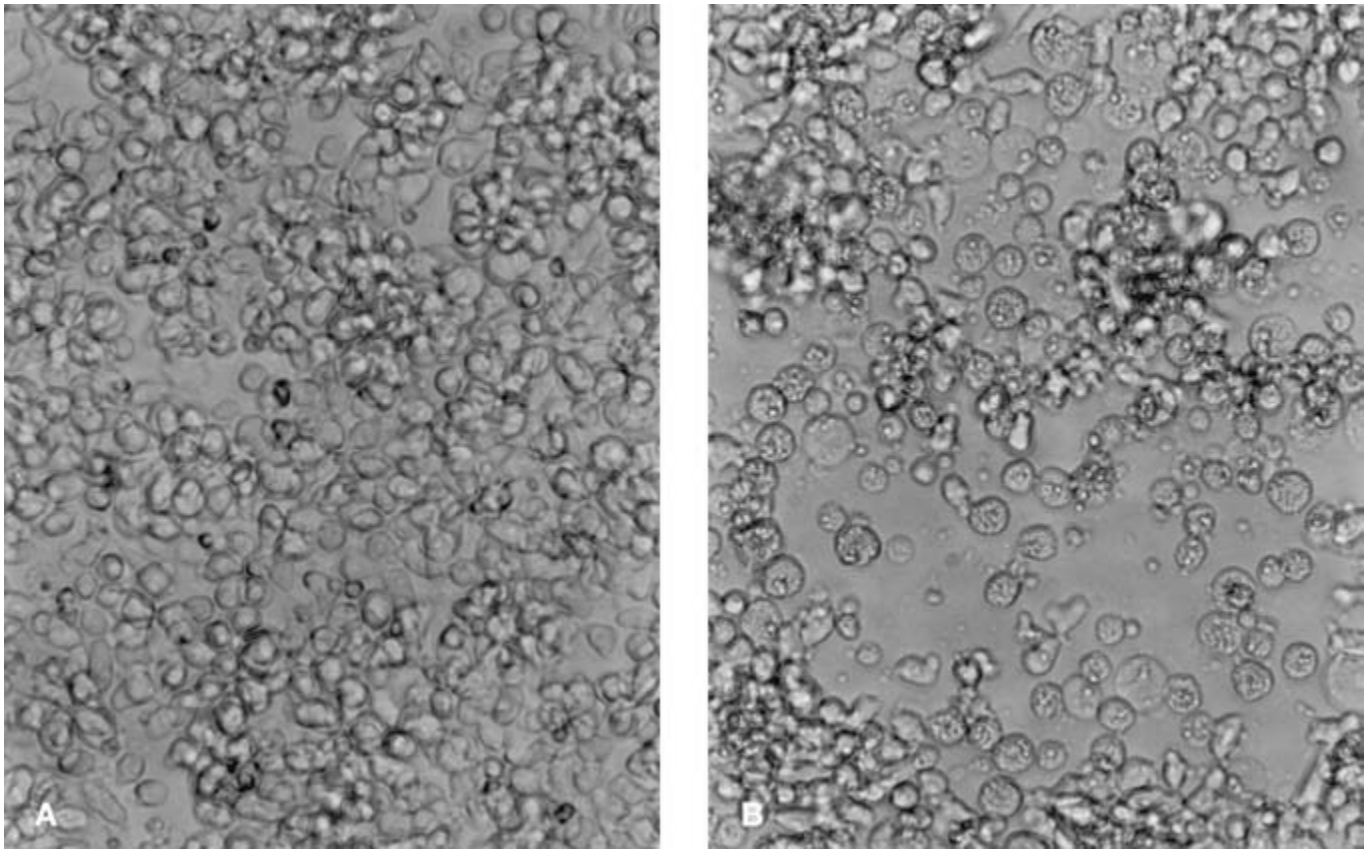
DNA-based Detection of CIAV

Polymerase Chain Reaction-based Techniques

PCR assays have become the assay of choice for the detection of CIAV DNA in infected cell cultures, chicken tissues, archived formalin-fixed paraffin-embedded tissues, or vaccines (145, 188, 200, 201, 213). The test proved to be specific and definitely more sensitive than cell-culture isolation of the virus and facilitates sequence and restriction enzyme analysis. Very high sensitivity is achieved with a nested PCR, which, however, is also most sensitive to cross-contamination (20, 188). A hot-start PCR for CIAV is also highly sensitive; furthermore, the use of a spike DNA as an internal control enables the validation of CIAV-negative samples and an estimation of the number of CIAV genomes present in the tested samples (44). Competitive PCR assays using a template with a deletion of 33 nucleotides allow quantitation of viral DNA (245). Real-time PCR assays for the quantitation of viral DNA and RNA have also been developed (108, 223). Different primers and conditions have been used successfully by different laboratories. For routine assays primers are best selected from the conserved ORF regions. DNA can be extracted from the same tissues as used for virus isolation. Miller *et al.* (130) used nested PCR to screen embryonal tissues and egg membranes obtained after hatching to analyze the presence of viral DNA in offspring of SPF hens. *In situ* PCR assays have been used to detect CIAV infected cells in the absence of detectable levels of VP3 (20).

DNA Probes

Detection of CIAV in formalin-fixed, paraffin-embedded thymus sections by *in situ* hybridization using a biotinylated DNA probe prepared by PCR (6, 136) or a digoxygenin-labeled cloned CIAV genome probe (43) has been described. Microwave treatment combined with protease treatment significantly enhanced the sensitivity of the assay (114). Biotinylated DNA probes have been used successfully to rapidly diagnose the presence of CIAV in blood smears (146, 175). Dot-blot hybridization assays using cloned ^{32}P-labeled DNA probes can detect viral DNA extracted from chicken tissues from 5 through 42 days after infection (208) or from MSB1 cells infected with field isolates of CIAV (145). A competitive hybridization assay conducted in 96-well plates utilizing a biotinylated CIAV DNA probe detects CIAV in DNA prepared from buffy coat cells of infected chickens from 3–28 days PI and can be used as a quantitative assay (147). While less sensitive than virus isolation from buffy coat cells, the com-



8.6. Cytopathic effect in cultured MSB1 cells 2 days after infection with chicken infectious anemia virus. A. Uninfected cells. B. Cells infected with a high dose of virus. Unstained. ×230. (von Bülow)

petitive hybridization assay has 100% specificity and has sensitivity similar to *in situ* hybridization of blood smears and dot-blot hybridization of buffy coat samples using biotinylated probes (147).

Detection of CIAV by Antibodies

Viral infection can be demonstrated in chicken tissues by immunofluorescence or immunoperoxidase staining. Thymus tissues, collected at 7–12 days after infection, are usually preferred for diagnostic tests. Tissue impression smears and cryostat sections, fixed with acetone, are used for either indirect or direct immunofluorescence staining employing polyclonal chicken or rabbit hyperimmune serum or MAb to CIAV (75, 78, 116, 124). Immunoperoxidase assays are performed with formalin-fixed, paraffin-embedded or frozen sections (78, 116, 185). Pretreatment of tissues with proteases III or XIV greatly enhances the detection of viral antigens (116). The most satisfactory results are obtained with MAb, because polyclonal antibodies may produce a high level of nonspecific background staining.

Electron Microscopy

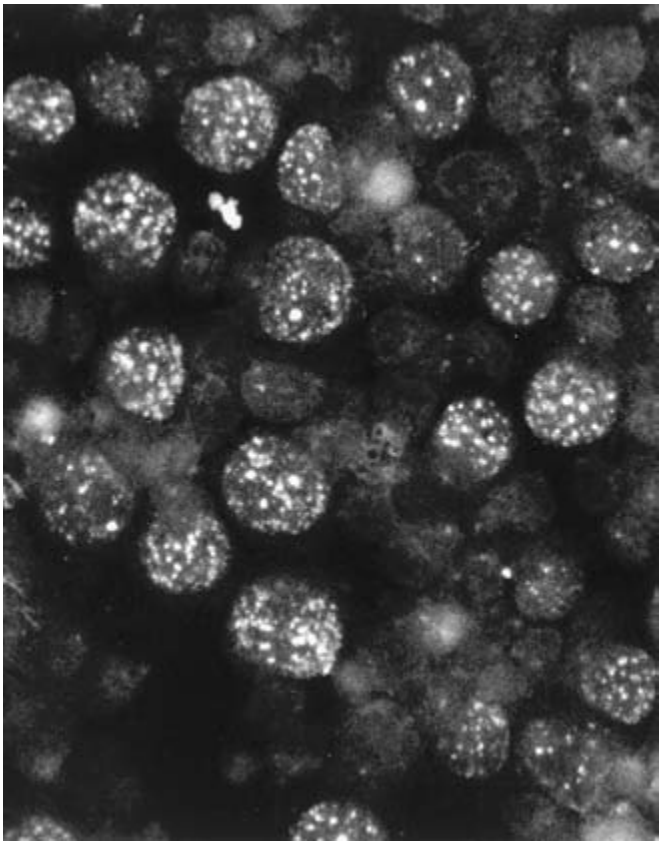
Electron microscopy for routine diagnostic examination for CIAV is not recommended due to its lack of sensitivity.

Serology

Three serological assays are routinely used: ELISA-based assays, indirect immunofluorescence assays, and virus neutralization (VN) tests. Selection of a given test depends on the purpose of the serological examination and costs associated with each assay.

Indirect Fluorescent Antibody Tests

The indirect fluorescent antibody (IFA) test (120, 234, 254) for the detection of antibodies is a standard IFA test. CIAV-infected MSB1 or CU147 cells are used as the source of antigen. Cells are collected just before the beginning of cell lysis, usually 36–42 hours after inoculation, smeared on glass slides and acetone fixed. Fluorescent staining of rather small, irregularly shaped granules in the nucleus of enlarged cells (Fig. 8.7) is considered evidence for antibody in the test serum. The concurrent appearance of fluorescent, somewhat irregular circular structures is also specific, but less frequent. This pattern of immunofluorescence is considered typical of tests with neutralizing CIAV antibody (14, 124, 211). Positive and negative reference sera should always be included in FA tests. Noninfected cells can be used as a control. Sera may have antibodies against MDV, and CU147 and MSB1 cells can express MDV antigens in the nucleus and cytoplasm, which may cause some confusion. Nonspecific staining and background staining



8.7. Chicken infectious anemia virus antigens detected by immunofluorescent staining in cytospin preparations of MSB1 cells harvested at 40 hours postinoculation. Antigens are seen in enlarged cells with characteristic intranuclear granular fluorescence. ×400. (von Bülow)

masking specific reactions can be largely reduced by using sufficiently diluted test sera, i.e., 1:40 to 1:100 or even more (120). Nonspecific staining due to direct binding of anti-IgG conjugates (106) may be controlled by selection of pretested conjugates.

Enzyme Immunoassays

Various commercially available enzyme-linked immunosorbant assay (ELISA) techniques for the detection and measurement of CIAV antibodies in chicken sera have been developed. These tests are routinely used to screen breeder flocks in countries were vaccines are available (46), but false-positive responses have been reported (128). Antigens are generally prepared from partially purified virus preparations grown in MSB1 cells, which may include MDV antigens. Recombinant technology has been used to produce VP3 as a fusion protein in bacterial systems (157) or VP1, VP2, and VP3 in baculovirus systems (85). VP3 and VP2, but not VP1, proteins could be used as ELISA antigens. Unfortunately, these antigens will not detect VN antibodies. However, Todd *et al.* (212) developed a blocking ELISA using a MAb, 2A9, that reacts with field isolates from different parts of the world and recognizes a VN epitope (124, 211). A similar assay, using the same neutralizing MAb, but an Australian CIAV isolate as antigen, has also been developed (197). The blocking assays have advantages in terms of costs (212) compared to the indirect assay described earlier (211) and yield fewer false positive results than commercial indirect ELISA kits that do not use a blocking format (197).

Virus-neutralization Tests

In the VN test (234, 258) serial two-fold dilutions of serum or egg yolk are mixed with equal parts of a CIAV suspension containing 200–500 $TCID_{50}$/0.1 mL, and the mixtures are incubated at 37°C for 60 minutes or at 4°C overnight before assay in MSB1 cell culture. Microtest plates are recommended if large numbers of sera have to be examined (83, 93). It may take up to 5 weeks, requiring eight to nine subcultures, before the assay is completed; however, results can be obtained much earlier, and subcultures can be omitted, if the virus concentration in the mixture is increased to $10^{5.0}$ to $10^{5.5}$ $TCID_{50}$/0.1 mL (153, 231, 234). In this instance, inoculated cultures should be examined microscopically for CIAV-specific CPE after both 2 and 3 days. One subculture may be required if complete destruction of virus-control cultures is desired to establish the endpoint. Subcultures can also be omitted if virus replication is assessed by PCR 3–4 days postinfection (223).

Qualitative VN tests for flock screening can be made with a constant serum dilution of 1:80–1:100 and a high dose of test virus as described above. Lower serum dilutions are not recommended because they can occasionally be cytotoxic or cause nonspecific inhibition of the virus. This type of test can be rendered semiquantitative by making a series of subcultures; the relative antibody level is indicated by the number of subcultures in which the inoculated cells stay alive (231, 234).

Direct comparisons among an ELISA test, IFA assay, and VN test were made by Otaki *et al.* (153). The VN test was more sensitive than the other two assays and the IFA assay frequently gave false-positive results, especially when sera were tested at dilutions of < 1:50. Unfortunately, no reports compare commercial ELISA kits with VN assays.

Differential Diagnosis

Infection criteria have only limited value in diagnosis of CIAV-induced disease, because CIAV is virtually ubiquitous among chickens. Demonstration of the virus, viral antigens, or viral DNA may be considered etiologically significant if detected at sufficiently high levels in a high proportion of affected birds. In chickens under 6 weeks of age, a typical combination of signs, hematologic changes, gross and microscopic lesions, and flock history are suggestive of CIA. However, no particular lesions can be considered pathognomonic.

Aplastic anemia, but not a pancytopenia, with a concurrent atrophy of thymus and bursa of Fabricius, and depressed immune response also can be caused by osteopetrosis virus. Anemia induced by erythroblastosis virus can be distinguished from CIAV-induced anemia by microscopic examination of blood smears. MDV can cause severe atrophy of the thymus and bursa of Fabricius, especially after infection with very virulent viruses (16, 129). IBDV induces atrophy of lymphoid tissues with typi-

cal histologic lesions but normally does not affect the thymus. MDV and IBDV normally do not cause anemia, although anemia has been described with some strains of MDV (54). Aplastic anemia that may be associated with acute IBDV occurs and disappears much earlier than CIAV-induced anemia (148). Adenovirus is a major cause of an inclusion body hepatitis-aplastic anemia syndrome that occurs most frequently between 5 and 10 weeks of age (29). It does not, however, induce aplastic anemia after a single infection of experimental chickens.

Intoxication with high doses of sulfonamides, or mycotoxins such as aflatoxin, can result in aplastic anemia and "hemorrhagic syndrome." Aflatoxin also may impair the immune system. In the field, however, chickens are rarely exposed to doses of aflatoxin or sulfonamides that are sufficient to cause acute disease. On the other hand, subclinical intoxication of chickens might add to the pathogenicity of CIAV or *vice versa*.

Intervention Strategies

Management Procedures

Attention should be paid to management and hygiene procedures to prevent immunosuppression by environmental factors or other infectious diseases and to prevent early exposure to CIAV. Improved hygiene has reduced seroconversion rates, but this may cause problems when flocks get exposed later in life (46, 95, 112). Eradication of CIAV is virtually impossible under field conditions and may be difficult on infected SPF premises. In the latter case, this is not only because of the high resistance of CIAV to disinfection, but also because viral DNA can be transmitted vertically, which may be reactivated during the laying cycle (20, 130). Monitoring of breeder flocks for the presence of CIAV antibody should be done to avoid vertically transmitted disease outbreaks or to test the efficacy of vaccinations.

Vaccination

Current vaccine strategies are based on the prevention of vertical transmission and horizontal transmission of virus to very young chicks by immunization of breeder flocks and have been successful in reducing the incidence of anemia in young chicks (46). Artificial exposure of young breeder flocks was originally achieved by transfer of litter from CIAV-infected flocks or by providing drinking water containing CIAV-positive tissue homogenate. This method is still used in countries where vaccines are not available or where vaccines are not applied for economical reasons. However, these procedures are very risky with regard to hygiene and level of exposure and should be discouraged (228). Commercial live vaccines are available in several countries (193, 227, 229, 230). Vaccination should be performed at about 9–15 weeks of age, but never later than 3–4 weeks before the first collection of hatching eggs to avoid the hazard of vaccine virus spread through the egg. Vaccines can be applied in the drinking water or by injection in the presence of adjuvants. Although these live vaccines are considered safe and efficacious, further studies are needed to examine potential deregulation of cytokines as a consequence of vaccination.

Several strategies utilizing various combinations of natural exposure, monitoring for seroconversion, and vaccination of breeders are actually used in the broiler industry. Smith (183) recently surveyed 68 complexes of eight large vertically integrated broiler production companies across the United States to determine which strategies are most commonly used for broiler breeders. He found that the majority of operations relied on natural exposure. Half of the operations did not routinely test for seroconversion, but some of those operations vaccinated breeders in new or cleaned houses where natural exposure might not occur. Approximately one-third of the operations relying on natural exposure did routinely test for seroconversion and subsequently applied commercial vaccines to flocks that did not exhibit adequate seroconversion. Smith noted the relatively high cost of commercial live CIAV vaccines as the reason for reliance on natural exposure. However, approximately one-third of the complexes surveyed did routinely vaccinate all breeder pullets between 10 and 12 weeks of age. Males are not usually vaccinated.

Based on the negative effect of CIAV on the generation of cytotoxic T cells when infection occurs after maternal antibodies have disappeared (109) vaccination for broilers may also be necessary. Recently a vaccine has been licensed in the United States for use in one-day-old broilers. Although these live vaccines are considered safe and efficacious, further studies are needed to examine potential deregulation of cytokines as a consequence of vaccination.

An inactivated vaccine has been tested in SPF breeder hens. Vaccinated hens showed seroconversion and their offspring were protected against challenge (156). Unfortunately, viral titers in MSB1 cells are generally low (117) and therefore inactivated vaccines may not be cost-effective. Although recombinant vaccines expressing VP1 and VP2 are certainly possible (99, 141) these have not been licensed to date.

Treatment

No specific treatment for chickens affected by CIAV infection is available. Treatment with broad-spectrum antibiotics to control bacterial infections usually associated with CIA might be indicated.

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Circovirus Infection of Pigeons and Other Avian Species

Leslie W. Woods and Kenneth S. Latimer

Introduction

Definition and Synonyms

Viruses belonging to the family *Circoviridae* are the smallest pathogenic DNA viruses that have been identified and characterized in mammalian and avian species (106). There are currently two genuses in the family *Circoviridae*; *Gyrovirus* and *Circovirus* (50). Chicken anemia virus (CAV) was recently placed in the newly designated genus, *Gyrovirus*; porcine circovirus-1 (PCV-1), porcine circovirus-2 (PCV-2), and psittacine beak and feather disease virus (PBFDV or BFDV) remained as members of the *Circovirus* genus (50), and pigeon circovirus (PiCV) (48, 111, 112), canary circovirus (CaCV) (62, 108), and goose circovirus (GoCV)(93, 105) were recently added to the *Circovirus* genus (109). Circoviruses have been identified from additional avian species over the past decade. Because of lower costs and more efficient technology, phylogenetic analyses have been performed on most circoviruses that have been recovered, allowing for comparison with the current members of the *Circovirus* genus. Tentative species in the genus *Circovirus* include duck (DuCV) (30, 109), finch (FiCV) (88) and gull (GuCV) circoviruses (110).

Circovirus infections have been documented in more than 60 species of Old World, New World and South Pacific psittacine birds (106), and in nonpsittacine birds including racing pigeons (*Columba livia*) (27, 87, 89, 111, 112), Senegal doves (*Streptopelia senegalensis*) (60, 68), canaries (*Serinus canaria*) (25, 62), finches (*Poephila castanotis, Chloebia gouldiae*) (55, 88), geese (4, 9, 91, 93, 117), Pekin and mulard ducks (30, 95, Banda et. al. submitted 2006), ravens (*Corvus coronoides*)(98), pheasants (*Phasianus colchicus*) (102), a southern black-backed gull (*Larus dominicanus*) (110), ostriches (*Struthio camelus*) (18, 19), and starlings (*Sturnus vulgaris* and *Sturnus unicolor*) (36).

Previously proposed names for this family of circoviruses have included *Diminuviridae* and *Circodnaviridae*. These names were based on the small size of the virion and the circular configuration of the DNA genome, respectively. Ultimately, the International Committee on Taxonomy of Viruses designated the new viral family as *Circoviridae* on the basis of the circular configuration of the genome (46). Synonyms of circoviral-associated diseases in various avian genera have included psittacine beak and feather disease (psittacine birds), feather and beak disease (psittacine birds), French moult (budgerigars), black spot (canaries), young pigeon disease syndrome (pigeons), fading chick syndrome (ostriches) and runting syndrome (geese).

None of the viruses in the genus *Circovirus* have been propagated in a cell culture system which has limited the accumulation of knowledge concerning these viruses and their associated diseases and thwarted attempts at commercial vaccine production. Recognition of circoviral infections in production flock species such as geese, ducks, pheasants and pigeons has increased awareness of the disease and emphasized the need for more research on the avian circovirus diseases and investigation of the potential impact of circoviruses on the commercial poultry industries. Currently, disease management requires extrapolation of biological understanding of viral disease from the well-researched companion bird circovirus, beak and feather disease virus (BFDV), and the clinical disease it produces. Therefore, all avian circoviruses of the genus *Circovirus* will be discussed in this chapter.

Economic Significance

Psittacine beak and feather disease is one of the most frequently diagnosed viral diseases of psittacine birds and, therefore, has caused tremendous economic loss in the pet bird trade and aviculture industry. The economic significance of many of the nonpsittacine circovirus-associated diseases has yet to be ascertained. Ultimately, the economic importance of circovirus diseases will be based on the future elucidation of the clinicopathologic significance of these circovirus infections in various avian species and circovirus prevalence and global distribution. Circovirus infections in pigeons and geese have been demonstrated to be highly prevalent in some regions of the world and may have global distribution. Putative immunosuppression and developmental abnormalities have been associated with circovirus infections in pigeons (104, 111, 112), geese (93), pheasants (102) and ducks (30, 95). Therefore, circovirus infections would have a globally significant economic impact if there is a high prevalence of circoviral-induced clinical disease in countries with large production industries of these species (4, 9, 95, 117). In addition to the economic impact, BFDV could have devastating effects on avian populations as some endangered species such as the Cape parrot (*Poicephalus robustus*) and black-cheeked lovebird (*Agapornis nigrigenis*) are highly susceptible to disease (31, 38).

Public Health Significance

Thus far, experimental work with a small number of the circoviruses has not demonstrated infectivity across host orders. In addition, natural cases of atypical host infections with circovirus have not been reported to date. Therefore, the public health significance of avian circoviruses appears to be minimal. Phylogenetic analyses have shown no substantial homologies between members of the genus *Circovirus* and human TT virus (TTV) and TTV-like minivirus, previously proposed members of the family *Circoviridae* (54, 100). In fact, these viruses were recently placed in a new genus *Anellovirus,* which is currently not attached to an existing virus family (8).

History

Feather changes consistent with PBFD have been observed in wild Australian parrots since the turn of the century (66). However, the disease *per se* was not described in detail in Aus-

tralian cockatoos until the early 1970s (41, 66). The disease was named "psittacine beak and feather disease" by Perry in 1981 (61). Viral particles measuring 17–22 nm in diameter were associated with histopathologic lesions. A viral etiology for PBFD was first proposed by Pass in 1984 and later established by Ritchie in 1989 (60, 72). Since that time, circoviral infections have been reported in more than 60 species of psittacine birds (108). By the mid-1980s, pathologists recognized intracellular inclusions in the lymphoid tissues of pigeons that resembled those of PBFD (28, 85, 111, 112). Pigeon circovirus (PiCV) subsequently was identified by electron microscopy in 1993 in the United States (111) and South Africa (22) and was proposed as the newest member of the *Circoviridae* family in 1994 (112). Reports of PiCV followed from the United States (87, 112), Canada (58, 112), Australia (112), the United Kingdom (89), Germany (92), Belgium (101), France (1), and Italy (10). During the next decade, circoviruses were identified and characterized in numerous other companion/captive/production and free-ranging avian species (30, 36, 62, 93, 95, 98, 102, 110).

Etiology

Classification

Porcine circoviruses (PCV-1 and PCV-2), chicken anemia virus (CAV), beak and feather disease virus (BFDV), canary circovirus (CaCV), goose circovirus (GoCV), and pigeon circovirus (PiCV) are currently listed in the *Circoviridae* family in the Eighth Report of the International Committee for the Taxonomy of Viruses (109). Based on similar virion size and genomic data, PCV-1, PCV-2, BFDV, PiCV, CaCV and GoCV were placed in the genus *Circovirus.* Chicken anemia virus was placed in the new genus, *Gyrovirus*, based on CAV's larger virion and genome, and different genomic organization. Duck circovirus (DuCV), finch circovirus (FiCV) and gull circovirus (GuCV) are tentative species in the genus (109) and starling circovirus (StCV) and raven circovirus (RaCV) have been proposed members of the genus *Circovirus* of the family *Circoviridae*. Each member is distinct based upon pylogeny and genomic structure (5, 9, 30, 36, 48, 62, 98, 103, 105, 106, 117).

Morphology

Ultrastructure

Ultrastructurally, circoviruses appear as nonenveloped virions with spherical to icosahedral (T=1) structures containing 60 capsid protein molecules arranged in 12 pentamer clustered units (11) and no obvious surface structures (flat capsomeres). Negatively stained particles have a mean diameter of 14–20.5 nm (11, 53, 106).

Genomic Size and Density

The entire sequence of the BFDV genome was published in 1998 by Niagro *et al.* (56) and Bassami *et al.* (5). Both groups determined that PBFDV genome was composed of 1,993 nucleotide bases. Later, analyses of eight PBFDV isolates revealed BFDV genomic sequences ranging from 1992 to 2018 nucleotide bases (6). Variations of nucleotide size and identity were attributed to point mutations, deletions and insertions in coding and noncoding regions of the genome. Analyses of genomic sequences of numerous other avian species followed. Genome sizes of 2,037, 1,996, 1,898, 1,952, 2063, and 1,820–1,821 nucleotide bases were determined for PiCV (48), DuCV (30), RaCV (98), CaCV (62), StCV (36) and GoCV (9) respectively. The buoyant density of PBFDV in cesium chloride (CsCl) gradient is 1.378 g/cc (72, 109).

Chemical Composition

The DNA viral genome of members of the family *Circoviridae* is circular and single-stranded with a characteristic stem loop structure (53, 103, 106). Early work by Ritchie *et al.,* comparing several isolates of PBFDV from different species of psittacine birds, identified three major viral proteins with molecular weights 26,000, 23,000, and 15,000 daltons (73). Minor proteins with molecular weights of 48,000 and 58,000 also were observed. Later work, however, demonstrated only two viral proteins, 26,000 and 23,000, which are believed to represent the capsid protein and putative replicase-associated protein encoded by open reading frames C1 and V1, respectively (47, 48, 104).

Virus Replication

Because none of the avian circoviruses have been propagated successfully in long-term cell cultures or chicken embryos, little is known about their replication. At present, only two members of the family *Circoviridae* have been propagated in cell culture (53, 106) and provide much of the available information on replication. Chicken anemia virus (CAV) has been grown in MDCC-MSB1 cells derived from Marek's disease lymphoma, and porcine circovirus (PCV) has been grown in PK-15 cells (porcine kidney cell line). Due to the limited size of the genome, circoviruses are highly dependent on cellular enzymes for replication. Circoviruses typically replicate in the nucleus, produce intranuclear inclusions and probably depend on cellular proteins produced during the S phase of the cell cycle (106). Mitosis is needed for DNA to be taken up into the nucleus and replication likely occurs in targeted rapidly dividing cells such as the basal feather follicular epithelium, lymphoid tissues and intestinal crypt epithelium. Results of immunohistochemical studies of birds with natural BFDV infection suggest viral persistence or replication may occur within the intestinal tract and its associated organs (40). Replication of the genome is believed to occur via a rolling circle that originates at the stem-loop structure. A replication-associated protein is encoded within the open reading frame V1 of the viral genome (47, 48, 103, 106). This highly conserved region exhibits marked similarity with the putative replication-associated protein of plant nanoviruses and geminiviruses (5, 23, 56, 106).

Susceptibility to Chemical and Physical Agents

Chemical and physical stabilities have primarily been determined for the circoviruses that can be propagated in cell culture systems (CAV and PCV). As a group, circoviruses are environmentally stable (53, 106, 109). These viruses are relatively resistant to in-

activation by many common disinfectants, acidic environments (pH 3), ether, chloroform, and high temperatures (60° for 30 minutes; 70º C for 15 minutes) (106, 109). Ten percent iodine and hypochlorite are required to inactivate CAV following treatment at 37ºC for 2 hours (118). PCV tested at 20ºC and 10ºC under protein load (40% fetal calf serum in virus suspension) with various combinations and amounts of glutaraldehyde, formic acid, formaldehyde and glyoxilic acid required over 60 minutes for inactivation (115). BFDV still retains ability to agglutinate erythrocytes after incubation at 80º C for 30 minutes (67). Because innate resistance to deleterious agents makes viral inactivation difficult, a commercially produced, killed circovirus vaccine is not currently available (79, 80).

Strain Classification

Antigenicity

A comparative study of four different BFDV preparations from two different species of cockatoos, an Amazon parrot, and a peach-faced lovebird showed that all four isolates were antigenically related using rabbit anti-BFDV polyclonal antibody (73). However, the circoviruses that infect pigeons and Senegal doves are antigenically distinct from BFDV. Immunohistochemical staining using both polyclonal (rabbit origin) and monoclonal (murine hybridoma origin) anti-BFDV primary antibodies (78) did not demonstrate visible chromagen deposition in circovirus-infected pigeon tissues (111). Tissue suspensions from psittacine birds infected with BFDV agglutinated cockatoo erythrocytes, while tissue suspensions of feather, liver, kidney, and gastrointestinal tract from circovirus-infected doves failed to hemagglutinate erythrocytes from galahs (68). Anti-psittacine circoviral hemagglutination inhibition antibodies were not found in doves exposed to psittacine birds infected with BFDV or in doves inoculated with BFDV.

Immunogenicity or Protective Characteristics

Clinically normal birds exposed to BFDV were demonstrated to have higher titers than birds with active viral infections, suggesting that antibody is protective against both viral infection and the development of clinical disease (77). Preliminary work with BFDV also demonstrated that maternal antibody is protective (76). Chicks from vaccinated hens remained clinically normal when challenged with BFDV, whereas chicks from unvaccinated hens were susceptible to viral infection and development of disease. Very few inoculation studies have been performed thus far with circoviruses infecting other avian orders. Anti-psittacine circoviral hemagglutination inhibition antibodies were not found in doves exposed to psittacine birds infected with BFDV or in doves inoculated with BFDV (68), so cross protective immunity does not appear to develop in avian species exposed to non-host circoviruses. However, further detailed studies are needed to clarify this observation.

Genetic and Molecular Characteristics

Putative phylogenies, comparative sequence analysis, and geographic distribution suggest there is an evolutionary link between the animal circoviruses (PCV and BFDV) and the plant nanoviruses and geminiviruses (23, 56). It is speculated that the circoviruses evolved from plant nanovirus DNA after a host-switch to a vertebrate, coupled with recombination in which the sequence encoding part of the replicase-associated protein combined with DNA from a calici-like virus.

Circoviruses have circular, single-stranded DNA, utilize an ambisense genome, and contain two major open reading frames (ORFs) in opposite orientation. One ORF codes for a putative replication protein (ORF V1) and the other ORF codes for a putative coat protein (ORF C1). These ORFs initiate close to a small noncoding region containing the potential stem loop and nonanucleotide motif (TAGTATTAC) associated with initiation of rolling circle replication of the viral DNA (5, 106).

Early work with pigeon circovirus demonstrated that it was related to, but distinct from, BFDV using DNA *in situ* hybridization with a short (40-base), single-stranded, oligonucleotide probe and a long (1900 bp), double-stranded, PCR-generated DNA probe (111). Complete nucleotide sequencing of the columbid circovirus (CoCV)/pigeon circovirus (PiCV) by Mankertz *et al.* displayed 55% homology to the genome of BFDV and 34% and 36% homology to PCV types 1 and 2, respectively (48). Nucleotide sequence analyses have been performed on most of the newly described tentative members of the avian circoviruses. Based on the regions encoding the capsid and the replicase-associated proteins, canary circovirus (CaCV) was shown to be closely related to PiCV (58.3%) (62). The starling circovirus (StCV) is closely associated with CaCV (67%) (36). Goose circovirus (GoCV) is less closely related to BFDV than PiCV is to BFDV (105). Sequence analysis of DuCV demonstrated 60% sequence homology with GoCV, 44% with PiCV, and 39% with CaCV (30). RaCV has greatest homology to canary and pigeon circoviruses (98).

Early studies with BFDV isolates showed no evidence supporting a relationship between genetic variation and region, pathogenicity, antigenicity or other physicochemical characteristics (6). In contrast, later investigations have suggested a relationship does exist between viral strain and region, species-specificity and pathogenicity. Recent studies have demonstrated that phylogenetic variation exists between isolates that infect different species within avian orders, suggesting the existence of viral strains. The first evidence presented to suggest variation in clinical disease and species susceptibility was reported in 1993 (38). In this report, 100% mortality occurred in captive black-cheeked and Lillian's lovebirds (*Agapornis nigrigensis* and *A. lilianae*) whereas exposed Fischer (*Agapornis fischeri)* and peach-faced (*A. roseicollis*) lovebirds had transient feather abnormalities and recovered. Sequence diversity has been demonstrated with BFDV isolates. Strains that infect lories and strains that cause the acute form of disease in African greys (*P. erithacus*) are specific genotypes (69). Ritchie demonstrated three distinct BFDV lineages in New Zealand in cockatoos, budgerigars and lorikeets suggesting a genotypic association between virus and host (81). Therefore it is apparent that different psittacine circoviral strains can infect different psittacine species causing disease in some but not other individuals within a species, subfamily or order. Additionally, individual species are often infected by closely related strains, but

highly divergent strains also have been detected. Different strains were detected in one clinically ill bird, suggesting either infection with multiple strains of circovirus or genetic divergence of non-pathogenic strains that mutated into a pathogenic strain. The relationship between viral strains, the avian species they infect and the pathogenicity of the strains is complex, however, and definite conclusions cannot be drawn at this time as to the nucleotide sequence of the genome of the infecting virus and its ability to cause disease (14).

The association between strains and the regions in which they are found has been investigated. Phylogenetic variation has been demonstrated between isolates from different regions. For example, variation exists between isolates of GoCV from Germany, Taiwan and China (4, 9) and between BFDV isolates from New Zealand, Australia and South Africa (31). South African BFDV isolates were shown to have diverged from viruses in other parts of the world with 8.3–10.8% diversity between African isolates and Australian isolates. In contrast, similarities exist between Australian and New Zealand circoviral isolates suggesting evolution of genotypic association between viruses and hosts predates dissemination throughout the world (81). There is no evidence of adaptive selection within BFDV populations (variation may be attributed to genetic drift).

DNA sequence analysis of 10 PBFDV isolates demonstrated that the sequence encoding the replicase-associated protein is highly conserved (116). Variation of findings evaluating the association of genotypes and factors such as region, host specificity and pathogenicity may be related to the region or length of the genome compared. Some investigations have evaluated variable lengths of the highly conserved replication-associated protein (ORF V1). Other studies have examined the ORF C1 region of the genome encoding the capsid protein which may more accurately reflect virus-host interaction.

Pathogenicity

Different clinical responses of different avian species to exposure and infection with circoviruses may be associated to numerous factors such as age of the bird at the time of exposure, natural resistance of individuals and species, and the strain of virus. The relationship between viral strains, the avian species infected, the clinical response, and the pathogenicity of the strains is complex and definite conclusions cannot be drawn at this time. (14)

The pathological significance of avian circoviruses has yet to be elucidated. There is substantial histologic and clinical evidence that immunosuppression is associated with circoviral infection in avian species (41, 43, 70, 71, 79, 80, 93, 104, 106, 110, 111, 112). Detailed evaluation of humoral and cell-mediated immune function following experimental infection with BFDV or PiCV has not yet been performed.

Pathobiology and Epidemiology

Incidence and Distribution of Disease

Psittacine beak and feather disease has worldwide distribution (41). The disease has been described in captive and wild populations in Australia (6, 51, 60), New Zealand (81), Africa (2, 31, 38, 39), Europe (7, 32, 63), North America (13, 58, 112) and Asia (37, 57) and is likely present in most regions of the world. The seroprevalence of BFD in different flocks of free-ranging psittacine birds in New South Wales ranged from 41–94% (66). In a 1993 study in which 10,000 captive birds in the United States were evaluated using a DNA probe test, 5% of birds tested positive for BFD viral antigen in blood (13). The majority of these birds did not have clinical signs of BFD, suggesting many birds may be subclinically or transiently infected with BFDV. Old World psittaciformes showed the highest incidence of positive tests. Eclectus parrots had the highest incidence of viremia (10.2%) followed by cockatoos (8.7%) and African grey parrots (8%). New World psittaciformes exhibited a much lower incidence of positive test results. Positive test results in lovebirds exceeded 30%.

Pigeon circovirus is distributed over a wide geographic area encompassing the United States (111), South Africa (22), Canada (58, 112), Australia (68, 112), and Europe (89, 92, 94, 101). The general practice of intermixing young, susceptible pigeons coupled with fecal shedding (27) and extreme persistence of the virus in environment (53, 80) may suggest widespread PiCV-infection of racing pigeons in all continents. Goose circovirus is highly prevalent in goose flocks in Hungary, Taiwan and China (4, 9, 117). In Hungary, GoCV was detected in 48% of 214 diseased or dead birds tested, representing 65% of 76 flocks tested (4).

Natural and Experimental Hosts

Beak and feather disease has been reported in more than 60 species of Old World, South Pacific, and New World psittacine birds with the highest prevalence in Old world and lowest prevalence in New World psittacine birds (106). Infection has been documented in both free-ranging and captive birds. Viral infection may cause acute to chronic, debilitating disease resulting in death. A few spontaneous clinical recoveries have been observed in budgerigars (*Melopsittacus undulatus*), lorikeets, and lovebirds (*Agapornis* sp.) with previous lesions of PBFD (38, 41). Pigeon circovirus infects racing pigeons (*Columba livia*) and Senegal doves (*Streptopelia senegalensis)*. It is currently unknown whether the PiCVs from pigeons and doves are genetically identical. Reports of natural circoviral infection in other avian species include infection of canaries (*Serinus canaria*) (25, 62), finches (55, 88) (*Poephila castanotis, Chloebia gouldiae*), geese (4, 9, 93, 117), ostriches (*Struthio camelus*) (18, 19), pheasants (102), Australian ravens (*Corvus coronoides*)(98), ducks (30, 95, Banda *et al.* submitted 2006), a western scrub jay (Woods, unpublished) and a southern black-backed gull (*Larus dominicanus*) (110). Experimental inoculation studies with avian circoviruses have been frustrated by the inability to replicate the avian circoviruses *in vitro*. Experimental inoculation studies have successfully reproduced disease in psittacine birds infected with virus purified from feather pulp homogenates (77, 114). Experimental inoculation of doves with BFDV has not produced clinical disease or an antibody response to the virus (68).

Age of Host Commonly Affected

Circoviral infection is typically reported in young birds (<3 years of age in psittacine birds and < 1 year of age in pigeons), but has

been reported to occur in birds as old as 20 years of age without previous clinical signs of disease (41, 71). This latter observation indicates that adult naïve birds may become infected and develop clinical disease, although subclinical or latent infection at a younger age or an extended incubation period of disease cannot be discounted. It is generally agreed, however, that birds are susceptible to infection prior to involution of the bursa. In single case reports of clinical circoviral disease in geese (2–9 wk old) (93), ducks (6 wk old) (95), ostriches (<8 wk old) (18), finches (3–6 mo old) (55), canaries (10–20 day old) (25), pheasants (10–30 days old) (102), and in a gull (bursa present) (110) the age of the affected birds were noted as neonates or juveniles. Subclinical infections with circoviruses have been demonstrated to be highly prevalent in both young and adult birds; however, younger birds have a higher rate of infection. In one study, 101/1516 apparently healthy parrots tested positive for BFDV by PCR. Furthermore, the young birds had a higher positive test result rate (7). In another study of clinically healthy psittacine birds, 58/146 tested positive for BFDV (63). In two other studies, 13/20 healthy older pigeons (1–9 years) tested positive by PCR (17) and another study using a PCR-based test detected circovirus in 45/50 healthy adult pigeons (20).

Transmission, Carriers, and Vectors

Neonatal budgerigars and galahs experimentally infected through the combined oral, intracloacal, and intranasal routes developed clinical BFD (75, 79, 80). Additionally, circovirus was demonstrated in crop secretions, feces, and feather dust of BFDV-infected psittacine birds. Results of this study suggest that aerosolized viral particles or direct ingestion of contaminated materials may account for natural routes of viral exposure. Transmission of PBFDV most commonly occurs through shedding of virus in feather dander, followed by fecal shedding, and feeding of chicks with regurgitated crop contents. Hess demonstrated that virus could be detected most frequently in the feathers which did not correlate with clinical signs, suggesting this may be where the virus persists and is shed by adult birds (32). Direct inoculation of the bursa via the cloaca has been suggested as another mode of viral transmission to psittacine birds in nests contaminated with feces and feather dander (65). Circoviruses have been demonstrated by direct electron microscopic examination of negatively stained preparations of intestinal contents from circovirus-infected pigeons, suggesting that shedding and transmission of virus may occur through ingestion or inhalation of feces-contaminated materials (22, 111). In another study by Duchatel *et al.*, PiCV could be demonstrated in 13/20 apparently health pigeons that ranged from 1–9 years of age (17). Viral DNA was detected most commonly in the respiratory organs (trachea, pharynx, lung) followed by spleen, kidney and liver. DNA also was detected in 8/22 embryos supporting vertical transmission. Crop washings, which have been suggested as source of infection in pigeons, were tested by Duchatel. Of 64 crop specimens that were tested, none were positive for circovirus (16). The PCR test results on blood and intestinal contents from healthy pigeons were increasingly positive with the age of the pigeon in one study (20). This observation indicates that the major direction of transmission is likely horizontal. Similar results were obtained by Duchatel in which cloacal swabs from 15.8 % of the birds were positive at 37 days of age and 100% at 51 days of age, suggesting that pigeons probably became infected in the rearing loft through horizontal transmission of the virus (17). Vertical transmission has been suggested in psittacine birds, pigeons and ostriches with circovirus infection. Chicks derived from artificially incubated eggs of a BFDV-positive Little Corella (*Cacatua sanguinea*) hen consistently developed PBFD after hatching (41). Additionally, 1-day-old pigeons had microscopic lesions compatible with circoviral infection that suggested probable vertical transmission (58). Lastly, circovirus was demonstrated in livers from nonhatched ostrich chicks supporting the theory of vertical transmission in ostriches (18).

Incubation Period

The incubation period of PBFD has been determined experimentally and varies with species and age at the time of viral exposure. In two studies with BFDV, experimentally inoculated chicks developed clinical signs of disease 25–40 days post-inoculation (76, 114). Disease incubation for galah cockatoos (*Eolophus roseicapillus*) and sulphur-crested cockatoos (*Cacatua galerita*) was 3–4 weeks (65). There are a few reports of circoviral infection in older birds; 4–5 years old (83) and 10–20 years old (41), suggesting that either older naïve birds can become infected or these birds were infected as young birds with a prolonged incubation period and/or latency of infection (41, 71).

Clinical Signs

Morbidity and Mortality

Mortality and clinical signs in psittacine birds infected with BFDV are variable and dependent on the species of bird; age of the bird at the time of infection; possibly the viral strain, and presence or absence of concurrent viral, bacterial, fungal, or parasitic infections (41). In some species, infected birds generally survive less than 6 months, but supportive care can considerably lengthen survival time. Secondary infections account for 69% of deaths in birds infected with BFDV (41). Putative immunosuppression may be associated with hypogammaglobulinemia and virus associated infection and destruction of lymphoid tissue. Jacobson found that birds infected with BFDV had lower serum protein concentrations with lower prealbumin and gamma-globulin concentrations (33). Experimentally inoculated birds became acutely depressed and anorectic 4 weeks post-inoculation (76, 114). Progressive symmetric feather dystrophy and loss subsequently developed. Less frequently, beak and claw deformities also occurred. Raue and coworkers found that African grey parrots had two distinct, genetically diverse strains of circovirus (69). One strain was associated with the acute form of disease that had severe leucopenia and nonregenerative anemia without feather disorders as described by Donely and Schoemaker (15, 86). The other strain of circovirus was associated with feather disorders.

In most disease outbreaks, circoviral infection in pigeons is associated with high morbidity and low mortality. However, mortality in circovirus-infected pigeon lofts may range from 1–100%.

Overall mortality ultimately depends upon age of the birds and presence of concurrent infections, as is the case with PBFD (49, 70, 112, 113). A broad range of signs and severity of clinical disease is observed in infected pigeons. In one report, clinical signs were not observed in king pigeons ranging from less than 1 week to 6 weeks of age (58). Red blood cell values and concentrations of hemoglobin and total protein were unaffected by viral infection. White blood cell values were more variable but apparently did not correlate with the degree of bursal damage.

When circoviral infection spreads through a pigeon loft, the most commonly reported clinical signs are poor performance, diarrhea, and ill thrift. Spontaneous clinical recoveries have been observed in some pigeons with clinical circoviral disease (112, 113).

Loss of flight and tail feathers was the only clinical sign reported in *Streptopelia senegalensis* (laughing turtle doves) with circoviral infection (60). Association between a clinical syndrome in ostrich chicks called fading chick syndrome and circoviral infection was demonstrated in 23/52 ostrich chicks with depression, weight loss, anorexia, and diarrhea prior to death (18)

Circovirus was detected in a canary with canary black spot disease and may be associated with this syndrome (25). Clinical signs in canaries with black spot include abdominal distension and failure to thrive. Chicks typically die within 7 days. Feather anomalies are not usually seen.

Clinical signs of circovirus infection in mulard ducks include feather dystrophy along the dorsum (hemorrhagic shafts) and poor body condition with low weight gain (30). Growth retardation and rearing losses between 10–70% have been reported (95)

A single case report of circovirus infection in a 10-wk-old finch described nasal discharge, dyspnea, anorexia and depression (88). In a separate report, circovirus virions were detected by electron microscopy in spleen from four 3–6 month old finches with feather loss over the neck, trunk and dorsal region (55).

Pathology

Gross Lesions

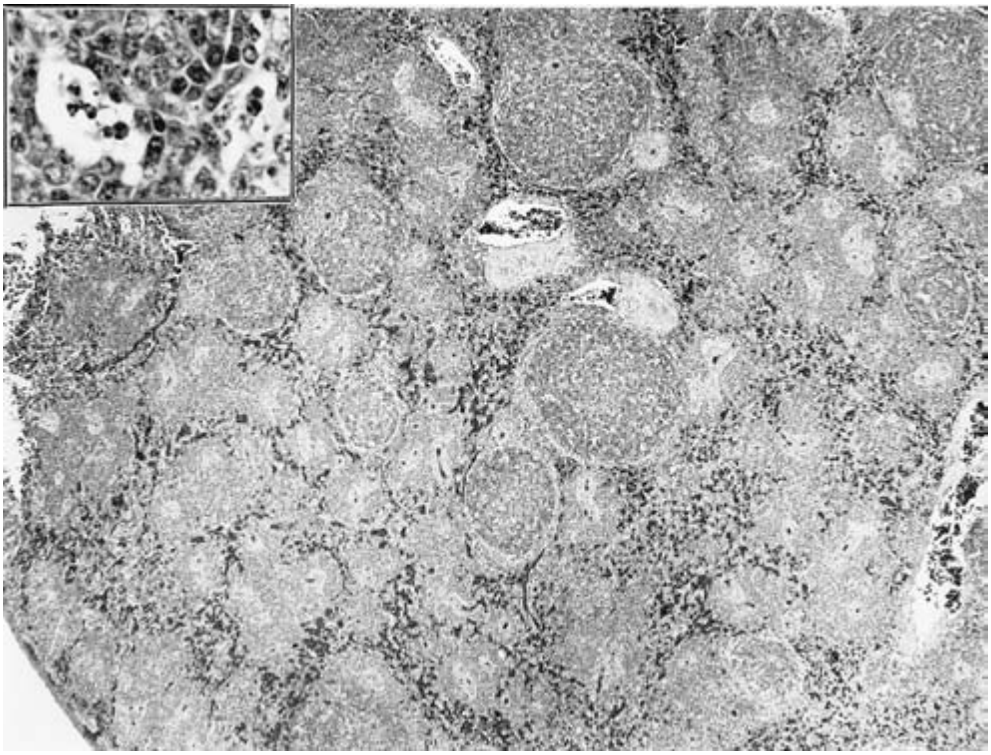
Feather loss in birds infected with BFDV may vary from subtle to severe, depending upon the stage of feather development when viral infection occurs (41, 59, 60, 114). Older birds may develop a chronic form of the disease in which the feathers stop growing shortly after emerging from the follicles. The number of dystrophic feathers increases during each successive molt. In some species, the powder down feathers over the hips are typically the first to show signs of dystrophy. The disease then progresses to involve the contour feathers in most tracts, followed by dystrophic changes in the primary, secondary tail, and crest feathers. Feather dystrophy and loss are roughly symmetrical. Changes in the feathers include retention of sheaths, hemorrhage within the pulp cavity, fracture of the proximal rachis, and failure of developing feathers to exsheathe. Short clubbed feathers, deformed curled feathers, stress lines within vanes, and circumferential constrictions may be observed. When present, beak deformities may occur including abnormal elongation, palantine necrosis, and transverse to longitudinal fractures or delaminations. Infrequently, the claws may develop deformities and the nails may slough.

Feather dystrophy is rare in racing pigeons with circoviral infection. The only gross lesion that can be attributed to circovirus infection in pigeons is bursal atrophy, but this lesion may not be present in all infected pigeons. The majority of the gross lesions observed at necropsy in pigeons that die of circovirus infection is usually attributable to secondary infections with other agents, such as concurrent viruses, bacteria, or fungi (112). However, there is a single report that describes feather dystrophy associated with an outbreak of circoviral infection in commercial pigeons in southern California (96). The gross appearance of the feathers was similar to that in psittaciform birds infected with BFDV. Symmetrical feather dystrophy and loss have been reported in Senegal doves with circoviral infection (60, 68). Feathering disorders also have been observed in finches (55), ravens (98), ducks (95) and geese (93) that were infected with circovirus. Canaries with circovirus infection or "black spot" have been reported to develop abdominal enlargement and gall bladder congestion (25). Necropsy findings in ostriches with fading chick disease include gastric stasis, yolk sac infection and enteritis (18).

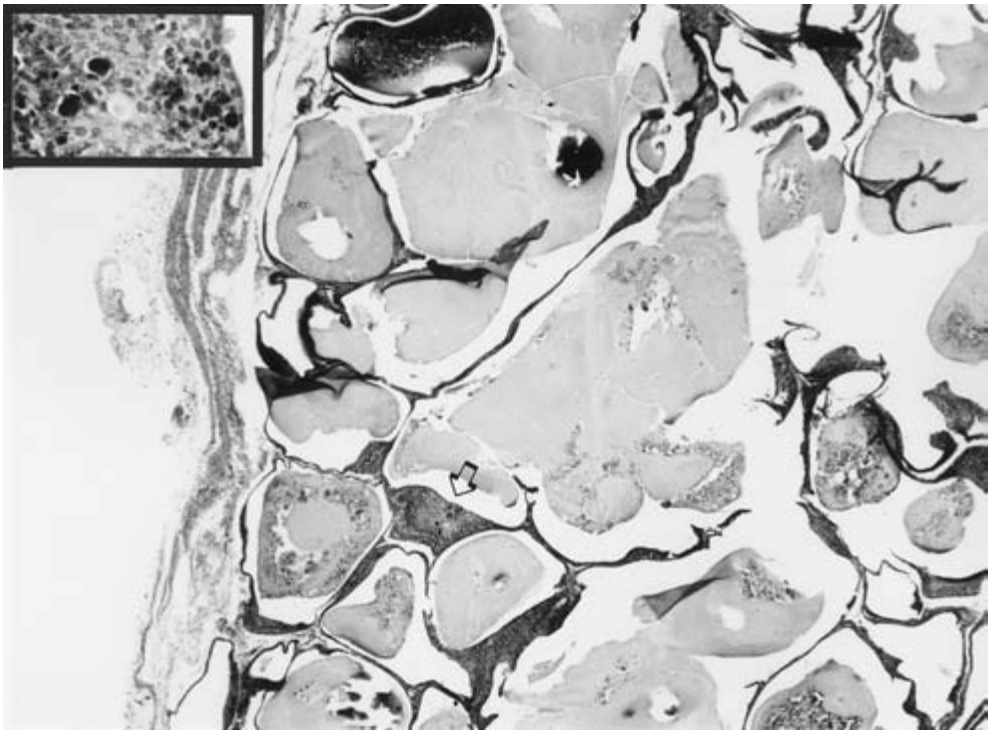
Microscopic Lesions

Variable degrees of necrosis and inflammation commonly are observed in dystrophic feathers of birds infected with circovirus (41, 59, 114). Similar lesions have been reported in Senegal doves (60, 68), commercial squabs (96), geese (93), ducks (95) and finches (55). Multifocal to diffuse necrosis is evident in the basal feather (pterlogenic) epithelium, and inflammatory cell infiltrates composed of variable combinations and quantities of heterophils and mononuclear inflammatory cells are seen with and without hemorrhage in the pulp cavity. Lesions also occur in the follicular epithelium but generally are less frequent and less severe. Basophilic to amphophilic nuclear inclusions are typically confined to feather and follicular epithelial cells, while multiglobular, "botryoid," or needle-like cytoplasmic inclusions are confined to macrophages in the feather epithelium, follicular epithelium, pulp cavity, and feather sheath. Associated degenerative changes occasionally are seen in the beak and claws. Cleft formation in the beak results from degeneration and necrosis of epithelial cells in the basal and intermediate cell layers of the rhamphotheca. These changes may be accompanied by hyperkeratosis and the presence of viral inclusions. In some psittacine birds such as African grey parrots, peracute infection with BFDV may be unaccompanied by feather and beak lesions; however, leucopenia, anemia (hypoperfusion of the lungs), bursal lesions and hepatic necrosis may be observed (86).

Changes in primary and secondary lymphoid tissues in birds infected with avian circoviruses may be a direct or indirect effect of circoviral infection. A broad range of microscopic changes may be observed in lymphoid tissues of circovirus-infected birds. These changes range from lymphofollicular hyperplasia with discrete lymphocellular necrosis to severe lymphoid depletion that is often accompanied by globular cytoplasmic viral inclusions within macrophages in the spleen, bronchial-associated lymphoid tissue, and gut-associated lymphoid tissue and within macrophages and bursal follicular epithelium. (Figures 8.8 and 8.9) (41, 112).

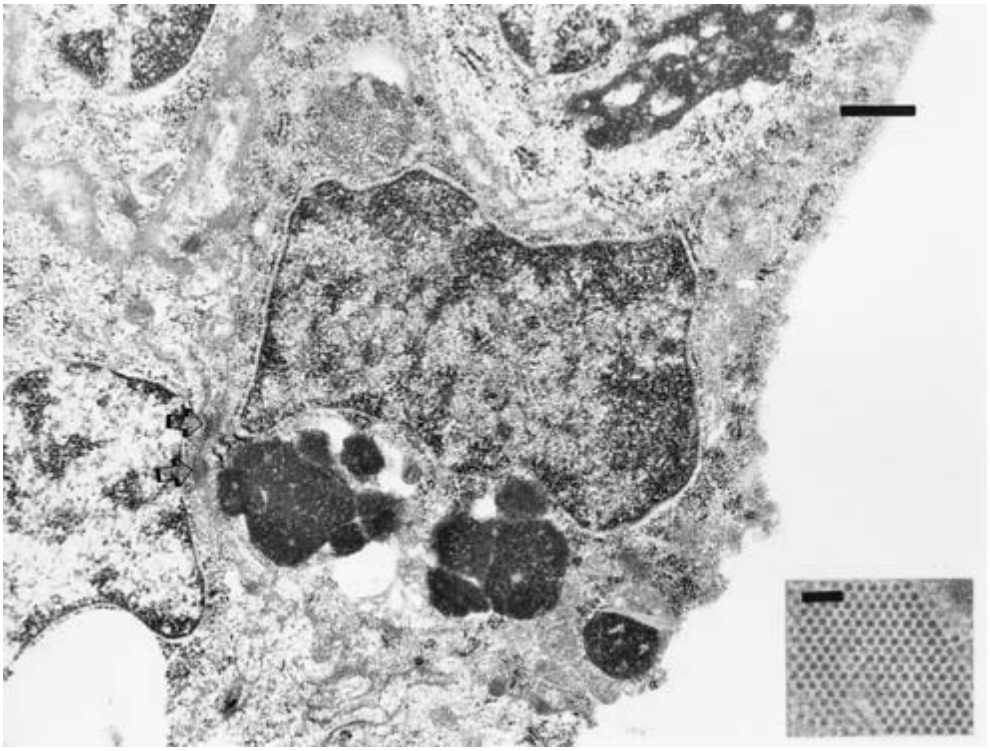


8.8. Photomicrograph of the spleen from a pigeon infected with circovirus. Note the lymphofollicular hyperplasia with discrete lymphocellular necrosis within the splenic follicles. H & E. Reprinted courtesy of *Journal of Veterinary Diagnostic Investigation*.



8.9. Photomicrograph of the bursa of Fabricius from a pigeon infected with circovirus. Note the severe cystic bursal atrophy. Basophilic "botryoid" cytoplasmic inclusions are present within cells in remnant bursal lymphoid tissue (*arrow*). Inset. Higher magnification of the cytoplasmic inclusions. H & E. Reprinted courtesy of *Journal of Veterinary Diagnostic Investigation*.

Microscopic lesions, other than those in cutaneous and lymphoid tissues, are generally dependent upon concurrent infections. One study that examined the extracutaneous distribution of viral inclusions in more than 35 naturally infected psittacine birds reported intracytoplasmic inclusions in macrophages of lymphoid tissues (bursa of Fabricius, thymus, bone marrow, spleen), beak, hard palate, esophagus, crop, tongue, parathyroid gland, liver, intestine, thyroid gland, adrenal gland, pancreas, and nail bed (40). Intranuclear inclusions were seen in epithelial cells in the intestine, beak, palate, esophagus, crop, nail bed, and testicular germinal epithelium. Inclusions were limited to the cytoplasm of macrophages in the thymus, tongue, parathyroid gland,



8.10. Transmission electron photomicrograph of the bursa of Fabricius from a pigeon infected with circovirus. The bursal epithelial cell has numerous cytoplasmic nonenveloped virions arranged in paracrystalline arrays. Arrows show desmosomes between epithelial cells. Bar = 500 nm. Inset. Higher magnification of virions (14–17 nm) forming a paracrystalline array. Uranyl acetate, lead citrate. Bar = 50 nm. Reprinted courtesy of *Journal of Veterinary Diagnostic Investigation*.

bone marrow, liver (Kupffer cells), spleen, thyroid gland, adrenal gland, and pancreas. Although multisystemic distribution of circoviral inclusions occurs in psittacine birds, this has not been reported in pigeons. However, demonstration of PiCV by *in situ* hybridization has demonstrated systemic distribution of circovirus which can be detected in liver, kidney, trachea, lung, brain, crop, intestine, spleen, bone marrow, and heart (90). *In situ* hybridization also has demonstrated viral distribution in the nucleus of cells in the sinuoids of the liver in ostriches with fading chick syndrome (18).

Ultrastructural Observations

Cytoplasmic inclusions within macrophages and bursal epithelium appear as relatively organized paracrystalline arrays, circles, or semicircles of viral particles (Figure 8.10) Infrequently, loosely arranged viral particles also may be observed in epithelial or endothelial cell nuclei. Virions typically measure 14–17 nm in diameter (83, 111, 112).

Pathogenesis

Feather dystrophy and beak and claw deformities can be attributed to circoviral-induced necrosis of the basal epithelium in psittacine birds with BFD. In one study, circovirus-infected birds had increased numbers of apoptotic cells in bursal lymphoid tissue as compared to uninfected birds undergoing physiologic apoptosis (1). The absence of demonstrated viral antigen within lymphocytes suggests that accelerated activation of apoptosis is not likely a direct effect of virus but an indirect cytokine-mediated event. Circoviral cytoplasmic inclusions are typically found in macrophages and bursal epithelium and correlate with phagocytic activity of infected cells rather than endogenously replicating virus.

The small circoviral genome with limited protein-encoding capacity depends on the host cell DNA replication machinery. Therefore, circovirus exhibits tropism for rapidly dividing cells in mitosis (106) such as basal follicular epithelium, lymphoid tissue and intestinal epithelium. Secondary infections account for approximately 70% of deaths in birds infected with BFDV, suggesting that viral infection results in acquired immunodeficiency (41). Putative immunosuppression and death from secondary infections also have been reported in pigeons (112), ducks (95), geese (93), a black-backed gull (110), and other avian species infected with circovirus (18, 104). Lymphocellular necrosis and lymphoid depletion in the bursa of Fabricius and spleen easily explain hypogammaglobulinemia, lack of humoral response to vaccination, and multiple concurrent infections that occur in circovirus-infected birds. Lymphocellular necrosis and depletion may directly impair both humoral and cell-mediated immunity. However, acquired immunodeficiency also may occur in circovirus-infected birds with microscopically normal-to-hyperplastic lymphoid tissues. In such cases, circoviral infection initially may target the monocyte-macrophage system, affecting antigen presentation and cell-mediated immunity. Studies designed to investigate immune function of experimentally inoculated birds will help to clarify the pathogenesis of circoviral infections.

Immunity

Active Immunity

Birds exposed to BFDV have been shown to seroconvert (64, 65, 67, 77). Clinically normal birds that have been exposed to BFDV

have higher titers than birds with active infections, suggesting that antibody is protective against development of clinical disease.

Passive Immunity

Experimental vaccination studies have demonstrated that chicks from vaccinated hens remain clinically normal following challenge with BFDV (76, 77). In contrast, chicks from unvaccinated hens succumbed to BFD when challenged with the same virus. These observations indicate that hens that have been inoculated with killed BFDV can transfer protective antibodies to their offspring (76, 77).

Diagnosis

Presumptive diagnosis of avian circoviral infection is based on clinical signs of disease and routine histology. The primary histologic finding is the presence of characteristic intranuclear and/or intracytoplasmic viral inclusions. Definitive diagnosis of circoviral disease requires the specific observation of characteristic virions by electron microscopy, demonstration of circoviral antigen by immunohistochemistry, or confirmation of circoviral nucleic acid using nucleic acid-based tests such as polymerase chain reaction (PCR), dot blot hybridization (DBH), nested PCR or *in situ* hybridization techniques (4, 7, 18, 20, 21, 29, 32, 44, 57, 69, 79, 82, 91, 107). The hemagglutination (HA) and hemagglutination inhibition (HI) test can be used to demonstrate BFDV antigen and antibody, respectively. Since circoviral infections can be highly prevalent and subclinical and latent infections can occur, demonstration of specific antibodies using hemagglutination inhibition indicates viral exposure but is not necessarily definitive for current viral infection. Additionally, erythrocyte suitability for HA/HI testing varies amongst species (84). Erythrocytes collected from many of the Australian and African birds will agglutinate in the presence of BFDV but there is no agglutinating activity using erythrocytes from South American species such as macaws and amazon parrots (which correlates with resistance of South American avian species to clinical beak and feather disease) (39). Additionally, HI is not effective for demonstrating antibodies in chronically infected birds.

In two separate studies, Smyth demonstrated that nucleic acid-based tests are more sensitive than histopathology at detecting infected birds. Of 107 pigeons examined by *in situ* hybridization, 89% were detected by PCR and 66% were detected by histopathology (90). Liver, kidney trachea, lung, brain, crop, intestine, spleen, bone marrow and heart were positive in circovirus-infected pigeons. Smyth suggested that when bursa of Fabricius is not available, liver is the next best tissue to examine for circovirus. Smyth also showed that routine histopathology was not adequate to detect GoCV infection in geese (91). Goose circovirus was demonstrated in bursa of Fabricius, thymus, bone marrow, liver, kidney, lung, heart and intestines by *in situ* hybridization.

Hess showed that feathers are the tissue of choice over cloacal swabs and blood to detect circovirus in infected birds, but that blood, rather than feathers, best correlates with clinical disease (32). Blood should be drawn from the vein and not the toenail due to possible environmental viral contamination. Toenail blood may be used to screen for circovirus infection, but if the test result is positive, blood should then be collected via venipuncture for confirmation testing (13). Hess suggests that the virus may persist in feathers of carriers (32). Newly erupted quills are the best samples of feathers to examine for viral infection (15). Duchatel tested pharyngeal swabs, cloacal swabs and blood immediately before sacrificing birds and found that these samples did not correlate with PCR results on tissues collected at necropsy (17). He concluded that these specimens are not good samples to take to eliminate birds with asymptomatic viral infection. Respiratory tract tissues most commonly tested positive followed by spleen, kidney and liver.

Nested PCR using DNA from dried blood collected by venipuncture was a very sensitive test and in fact was 10–100 times more sensitive than PCR (29, 37).

Todd (107) and Ball (4) demonstrated that dot blot hybridization (DBH) was less sensitive than PCR. Dot blot hybridization detects between 4 and 40 pg of viral DNA whereas PCR can detect 0.10 fg of viral DNA. Todd suggests that even though DBH is less sensitive than PCR, it is more useful for several reasons. Results of DBH are more likely to correlate with clinical disease if virus load is considered to be an indicator of disease. Higher circoviral titers likely correlate with clinical disease in avian species as is the case in pigs with PCV. DBH is semiquantative, cross contamination is not probable, and DBH is not limited by minor sequence variation like PCR.

Isolation and Identification of Causative Agent

Long-term propagation of the avian circoviruses in cell culture systems and embryonated chicken eggs has been unsuccessful. Only CAV has been propagated in MDCC-MSB1 cells derived from Marek's disease lymphoma.

Serology

Hemagglutination and hemagglutination inhibition have been used historically to confirm certain viral infections (hemagglutination) or to detect viral exposure by demonstrating viral-specific antibodies (hemagglutination inhibition). Ritchie *et al.* (77) and Raidal *et al.* (64, 67) have reported on BFDV-induced hemagglutination and the development of a hemagglutination inhibition test for detection of BFDV antibodies. These techniques have been used experimentally to partially elucidate the pathogenesis of BFD and to document the exposure of companion and free-ranging birds to BFDV. Erythrocytes from different avian species have variable hemagglutinating activity in response to circovirus. Therefore, validation of each assay system is necessary for accurate and precise diagnostic use.

Differential Diagnosis

Feather folliculitis (due to bacterial, fungal, or other viral infections such as polyomavirus), metabolic imbalance, endocrinopathy, and nutritional disease may result in feather dystrophy that mimics the clinical appearance of PBFD. Because secondary infections are common in circovirus-infected birds, circovirus should always be considered in conjunction with other infectious agents.

Intervention Stategies

Management Procedures

Prevention of circoviral infection is difficult in pigeon lofts and free-ranging bird populations where individuals of various age, origin, and species may intermix freely. Routine biosecurity and isolation procedures will be more effective in preventing viral spread in closed aviaries. After circoviral infection is established, disinfection of lofts, houses and aviaries is difficult to impossible. From results of studies with CAV and PCV, the circoviruses as a group appear environmentally stable and relatively resistant to inactivation by many common disinfectants (3, 53, 115).

Vaccination

Currently, an effective, commercial vaccine does not exist to prevent BFD in psittacine birds or circoviral infections in pigeons. Recombinant technology will be essential in developing effective vaccines, because none of the avian circoviruses (other than CAV) have been grown successfully in cell culture. Preliminary work with BFDV has shown that maternal antibody is protective (67, 77). Additionally, vaccination has been shown to be effective against the development of clinical disease but not against viral infection (65). Therefore, subclinical carriers are not prevented by vaccination. BFDV is extremely difficult to inactivate completely and is highly infectious to susceptible birds. Therefore, inactivated vaccines containing whole virus that is obtained from infected bird tissues should be considered too dangerous for clinical use (80). In one study (65), three sibling sulphur-crested cockatoos were vaccinated with inactivated vaccine but all three died of BFD prior to challenge with live virus. It is speculated that these birds may have been infected prior to vaccination, but the possibility of vaccine-induced disease was also considered.

Treatment

At the present time, an effective treatment does not exist for circoviral infections in psittacine or nonpsittacine birds. Because most birds infected with circovirus die from secondary infections with viral, bacterial, protozoal, or fungal agents, attempts should be made to diagnose and treat any secondary infections. Avian gamma interferon has shown some promise as part of a treatment regimen in birds with active infection. In one study (97), 7/10 African grey parrots (*Psittacus erithacus*) with active BFDV infection (PCR positive and severe leucopenia) responded to treatment with avian gamma interferon (Lowenthal;1 million IU IM SID x 90 d) coupled with quaternary ammonium (1:125; 15 minute, BID) nebulization. Leukocyte counts in surviving birds returned to normal 180 days after treatment was initiated.

Infection of macrophages and destruction of lymphoid tissues during viral infection undoubtedly affect both humoral and cell-mediated immunity. Therefore, birds should not be vaccinated while viral infection is active. Pigeons vaccinated with poxvirus and paramyxovirus vaccine during active circoviral infection in birds in the loft failed to elicit an antibody response to either agent (112). Therefore, antibody titers of birds in an aviary or loft that were vaccinated near or during the time of circoviral infection should be evaluated to determine the efficacy of vaccination. If postvaccinal serum antibody titers are low, revaccination should be performed after clinical signs of disease have subsided in the loft. Vaccination programs in aviaries should always remain current because viral infections are common, secondary viral infection may be concurrent with circovirus infection, and secondary viral infections often are responsible for increased mortality during circoviral outbreaks.

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Chapter 9

Adenovirus Infections

Introduction

Scott David Fitzgerald

Adenoviruses are common infectious agents in poultry and wild birds worldwide. Many of the viruses replicate in healthy birds with little or no apparent signs of infection, although they can quickly take on the role of opportunistic pathogens when additional factors, particularly concurrent infections, adversely affect the health of the avian host. Some adenoviruses however (e.g., turkey hemorrhagic enteritis virus, quail bronchitis virus, and egg drop syndrome virus) are primary pathogens in their own right, and others continually turn up in specific disease situations, indicating a degree of guilt by association, although the results of experimental infections to elucidate pathogenic intent have not always been successful.

The first avian adenovirus was isolated in 1949 when material from a case of lumpy skin disease in cattle was inoculated into embryonated chicken eggs (12). Other early unintentional isolates of fowl adenoviruses were the chicken embryo lethal orphan (CELO) isolates made in embryonated eggs (13) and the GAL viruses from chicken cell cultures (4). The first isolate of an avian adenovirus from diseased birds was from an outbreak of respiratory disease in bobwhite quail (*Colinus virginianus*) by Olson (11). Human adenoviruses were isolated in 1954 during investigations of respiratory disease (8) and initially were called adenoidal-pharyngeal-conjunctival agents, but the name adenoviruses subsequently was adopted (7).

Most of the viruses replicate readily in avian cell cultures derived from tissues, such as liver or kidney. Replication takes place in the nucleus and is accompanied by the development of intranuclear inclusions, which may aid histopathological diagnosis (9). The general properties required for classifying an isolate as an adenovirus have been defined by the International Committee on Taxonomy of Viruses (ICTV) (3). This report recognized two genera, *Mastadenovirus* and *Aviadenovirus,* within the adenovirus family, with human adenovirus type 2 and CELO virus as the respective type species (Table 9.1). The aviadenoviruses are serologically distinct from mastadenoviruses (10) and differ also in their genome organization (3). The genus contains most of the characterized adenoviruses isolated from chickens, turkeys, and geese (see "Group I Adenovirus Infections"), and these viruses often are referred to as group 1 avian adenoviruses in the literature (9).

However, two of the most important adenoviruses causing significant disease in avian species (hemorrhagic enteritis , referred to as group II [see section on "Hemorrhagic Enteritis and Related Infections"] and egg drop syndrome virus, referred to as group III [see subchapter on "Egg Drop Syndrome"]), show substantial differences at the molecular level from the aviadenoviruses. Currently, hemorrhagic enteritis (HE) virus, along with the related viruses of marble spleen disease (MSD) virus of pheasants and splenomegaly virus of chickens, and a recently isolated virus from a frog form the genus *Siadenovirus,* named to reflect one of their unique genome characteristics, namely the presence of a gene coding for sialidase (5, 6). Egg drop syndrome (EDS) virus, along with certain related adenoviruses isolated from ruminants, marsupials and reptiles, are now classified as members of the genus *Atadenovirus*, reflecting their high adenine-thymidine (AT) content (1, 2, 3, 6) (Table 9.1). In addition, a recently characterized adenovirus from a fish appears unrelated to currently recognized genera and may represent a fifth unnamed adenovirus genus (1, 6).

In this review, the group I, II, and III designations, which are commonly used in the literature, will be followed (9).

Table 9.1. Classification of adenoviruses.

Family: *Adenoviridae*
Genus: *Mastadenovirus* Mammalian adenoviruses
Human, simian, bovine, equine, murine, porcine, ovine, caprine, etc.
Genus: *Aviadenovirus* Group I Avian adenoviruses
Conventional adenoviruses of chicken, turkey, duck, and goose.
Five species A, B, C, D, and E; 12 serotypes
Genus: *Siadenovirus* Group II Avian adenoviruses Hemorrhagic enteritis virus (turkeys)
Marble spleen disease (pheasants)
AASV (chickens)
Genus: *Atadenovirus* Group III Avian adenoviruses
Egg drop syndrome virus and related viruses

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Group I Adenovirus Infections

Brian McConnell Adair and Scott David Fitzgerald

Introduction

Definition and Synonyms

The subgroup I avian adenoviruses comprise the genus *Aviadenovirus* within the adenovirus family. In contrast with the clear association of subgroup II (turkey hemorrhagic enteritis and related viruses, and subgroup III (egg drop syndrome) adenoviruses with disease, the role of most subgroup I avian adenoviruses as pathogens is not well defined. Notable exceptions include the FAdV-1 strains, which cause quail bronchitis (see the subchapter on Quail Bronchitis), and also the FAdV-4 strains, which play a major role in the etiology of hydropericardium syndrome. In addition, other strains rapidly can exploit opportunities presented when the health of the bird is compromised (e.g., by coinfection with other pathogens such as chicken infectious anemia virus [CIAV] or infectious bursal disease virus [IBDV]). Strains from several species, but particularly those belonging to species E (see discussion later in this chapter), have a particular predilection for growth in liver cells, and in certain circumstances that at present are not clearly defined can cause severe liver damage leading to the condition known as Inclusion Body Hepatitis (IBH) (see later discussion). Several aviadenovirus reviews are available (7, 71, 94, 96, 97, 99, 101).

Economic Significance

With such variability in their disease association, it is not possible at present to assess the overall economic importance of the subgroup 1 viruses.

Public Health Significance

There is no evidence of productive infection of human cells by subgroup 1 viruses, and therefore any public health implications are likely to be minimal. However there is growing interest in the use of FAdV-1 (CELO) as a gene transfer vehicle for use in humans and possibly other species (142). The virus has been shown to be able to transduce human cell lines without productive replication, and a CELO vector, encoding the herpes simplex virus type 1 thymidine kinase (TK) gene, has been shown to induce anti-cancer activity in human cells (142). Also, there are indications that the avian adenovirus SMAM-1 may play a role in human obesity, although this remains controversial at the present time (48).

Etiology

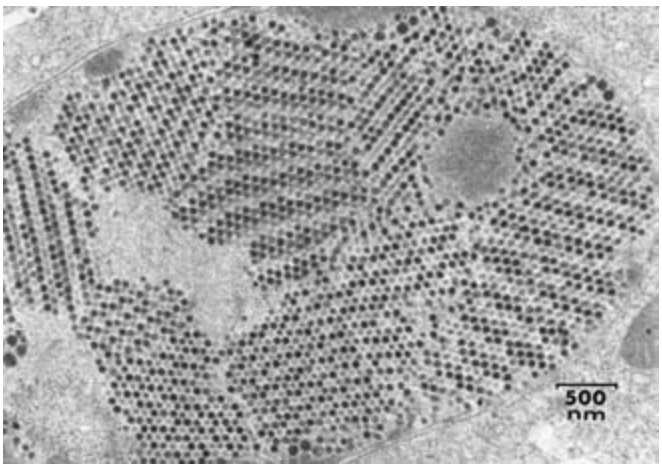
Classification

Within the adenovirus family, species designation depends on at least 2 of a number of key criteria that include calculated phylogenetic distance, restriction enzyme fragmentation, host range, pathogenicity, cross neutralization, and possibility of recombination (16). Five aviadenovirus species, designated with the letters A–E are recognized, based largely on molecular criteria in particular restriction enzyme fragmentation patterns and sequencing data (Table 9.2). Viruses within each species are further subdivided into serotypes based largely on the results of cross neutralization tests (23, 39, 42, 67, 80, 81, 83, 99, 102).

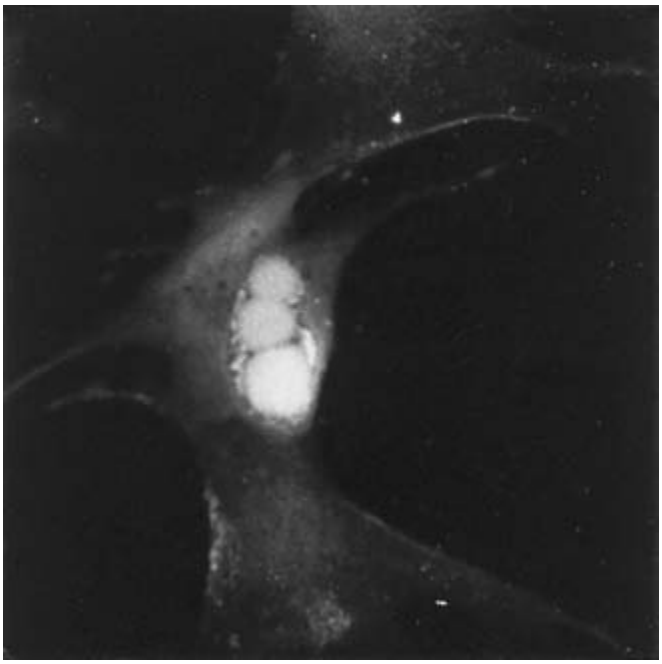
Table 9.2. Group 1 avian adenoviruses—classification.*
Genus *Aviadenovirus* (Group 1 avian adenoviruses)

Species in the Genus
Fowl adenovirus *A*
Serotypes: Fowl adenovirus 1 (FAdV-1) (CELO, 112, QBV, Ote, H1)
Fowl adenovirus *B*
Serotypes: Fowl adenovirus 5 (FAdV-5) (340,TR-22, Tipton, M2)
Fowl adenovirus *C*
Serotypes: Fowl adenovirus 4 (FAdV-4) (506, J2, KR5, H2, K31, 61)
Fowl adenovirus 10 (FAdV-10) (C-2B, M11, CFA20, SA2)
Fowl adenovirus *D*
Serotypes: Fowl adenovirus 2 (FAdV-2) (GAL-1, 685, SR-48, H3, P7)
Fowl adenovirus 3 (FAdV-3) (SR-49, 75, H5)
Fowl adenovirus 9 (FAdV-9) (A2, 90, CFA19)
Fowl adenovirus 11 (FAdV-11) (380, UF71)
Fowl adenovirus *E*
Serotypes: Fowl adenovirus 6 (FAdV-6) (CR119, 168)
Fowl adenovirus 7 (FAdV-7) (YR36, X-11, 122)
Fowl adenovirus 8a (FAdV-8a) (58, TR-59, T-8, CFA40)
Fowl adenovirus 8b (FAdV-8b) (764, B3, VRI-33)
Tentative Species in the Genus Aviadenovirus
Duck adenovirus (DAdV) (Duck adenovirus 2)
Pigeon adenovirus (PiAdV)
Turkey adenovirus (TAdV) (Turkey adenovirus 1, 2)

*(Based on Benko *et al*. [14])



9.1. Adenovirus-infected chick liver cell culture (48 hours post-infection). Adenovirus particles almost fill the nucleus.



9.2. Growth of FAdV-8 (764) in chick kidney cell cultures. Intranuclear inclusions stained by direct immunofluorescence.

Morphology

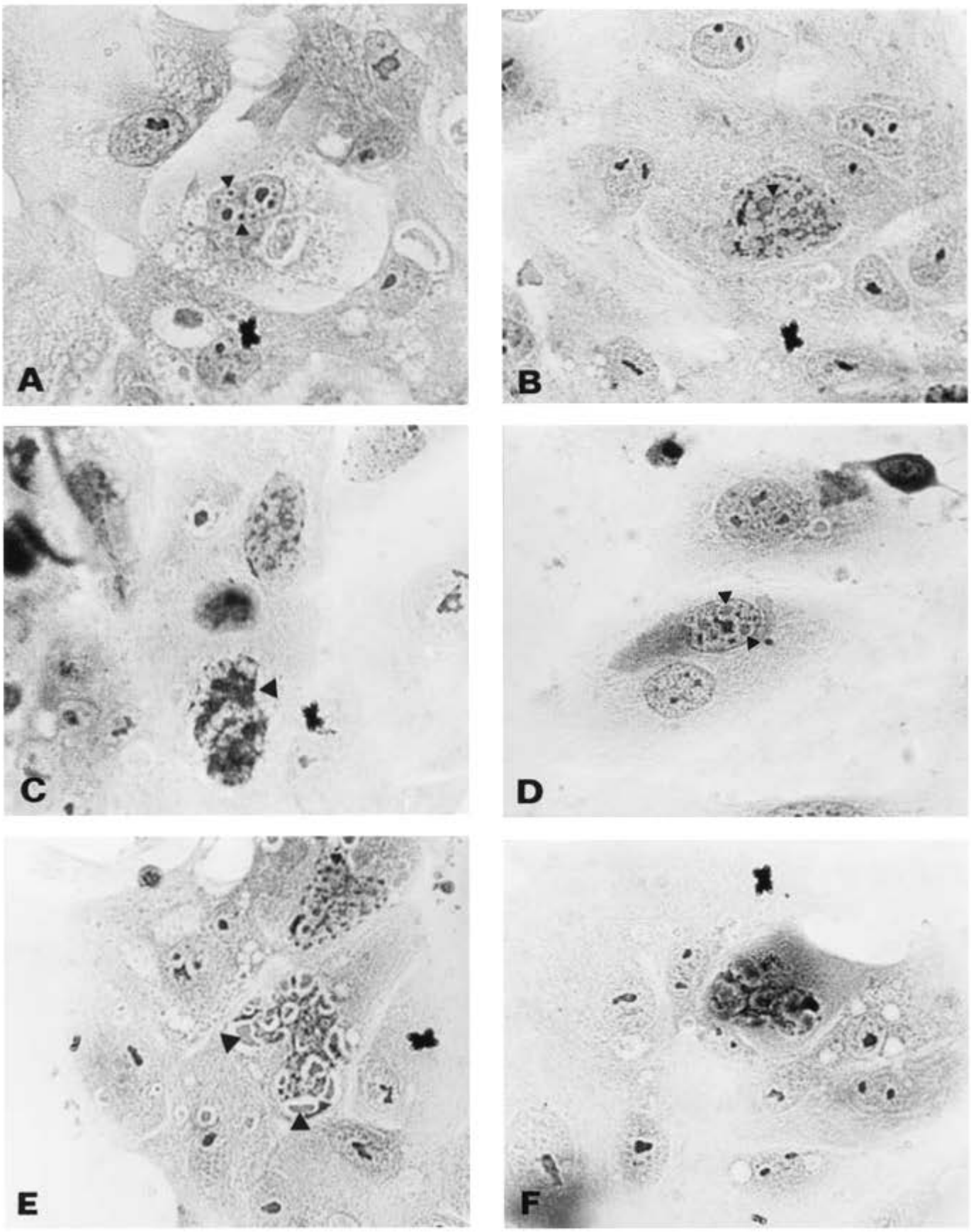
Ultrastructure and Symmetry

Details have been reviewed previously (94, 97). The virion is composed of 252 capsomeres, surrounding a core 60–65 nm in diameter. Capsomeres are arranged in triangular faces with six capsomeres along each edge. There are 240 nonvertex capsomeres (hexons) of 8–9.5 nm diameter and 12 vertex capsomeres (penton bases). Vertex capsomeres carry projections known as fibers (134). Mammalian adenoviruses have one fiber on each penton base, and the aviadenoviruses appear to have 2 (27, 59). In most cases, both fibers are of similar length, and there appears to be a relationship between fiber length and antigenic properties, because serotypes, which are related in cross-neutralization tests, have fibers of similar length. FAdV-1 (CELO), however, possesses 2 fibers of different lengths (i.e., 42.5 and 8.5 nm) which appear as 2 distinct proteins by partial peptide mapping (87, 89). Sequencing studies with FAdV-1 (CELO) have confirmed these findings and indicated the presence of 2 fiber genes (27). Exactly how the vertex capsomere is assembled to accommodate 2 fibers is not fully understood (27).

Ultrastructural studies demonstrate accumulation of virus particles in the nucleus of infected cells, and these often form crystalline arrays (3) (Fig. 9.1). Four types of inclusions, which differ in morphology and density depending on the content of viral protein and DNA, have been recognized (2,3). Large intranuclear inclusions are also clearly visible in tissues from infected birds or in infected cell cultures by cytochemical or immunostaining methods (Figs. 9.2 and 9.3), and these may be useful in diagnosis of adenovirus infections.

Size and Density

The adenovirus virion is a nonenveloped, icosahedral structure 70–90 nm in diameter (97). Densities between 1.32 and 1.37 g/mL in cesium chloride (CsCl) have been estimated for aviadenoviruses. Similar differences in density, which have been attributed to differences in DNA content and base composition in different isolates, have also been found in human adenoviruses.



9.3. H & E staining of chick kidney cells infected with FAdV-8 (768) showing basophilic inclusions in the nucleus.

Chemical Composition

The nucleic acid is double-stranded DNA, which accounts for 11.3–13.5% of the virion, with the remainder being protein (162). Others, however, have reported 17.3% DNA in FAdV-1 and estimated the guanine-cytosine (G-C) content of DNA at 54%, intermediate between the G-C contents of highly oncogenic (47–49%), and nononcogenic serotypes (57–59%) of human adenoviruses. Between 11 and 14 structural polypeptides have been described for FAdV-1.

Virus Replication

Adenovirus replication is divided into 2 well-defined phases. The early phase involves the entry of virus into the host cell and the transfer of the virus DNA to the nucleus, which is followed by the transcription and translation of the so-called early (E) genes (134). Proteins coded by the early genes are responsible for a redirection of cellular functions, in order to facilitate replication of the virus DNA and the consequent transcription and translation of the late (L) genes, coding for the virus structural proteins. Assembly of the viral proteins into complete virions is completed in the nucleus, and this is followed by disruption of the nuclear membrane and release of virus by destruction of the cell.

Susceptibility to Chemical and Physical Agents

All avian adenoviruses tested so far have shown typical adenovirus properties (see reviews 7,21,94,97,100). They are resistant to lipid solvents such as ether and chloroform, sodium deoxycholate, trypsin, 2% phenol, and 50% alcohol. They are resistant to variations in pH between 3 and 9 but are inactivated by a 1:1000 concentration of formaldehyde. They are inhibited by the DNA inhibitors IuDR and BuDR.

Although it is accepted that adenoviruses in general are inactivated in aqueous solution after exposure to 56°C for 30 minutes and that heat stability is reduced by divalent ions, the avian adenoviruses show more variability and are apparently more heat resistant. Some strains survive 60°C and even 70°C for 30 minutes. The infectivity titer of one FAdV-1 virus fell rapidly after 180 minutes at 56°C, and another FAdV-1 strain apparently survived 18 hours at 56°C. Even strains tested in the same laboratory have shown differences in thermostability, suggesting the differences were not due to variation in technique. Although most workers found that divalent cations destabilize adenoviruses, some have found no effect. These divergent results may be due to technique, and it is important to standardize suspending media and pH carefully.

Hemagglutination

Hemagglutination has been reviewed by McFerran (97). FAdV-1 virus hemagglutinates rat erythrocytes. Optimal agglutination of erythrocytes occurs between pH 6 and 9 at temperatures between 20° and 45°C. The hemagglutinin is stable to treatment with trypsin, RNase, DNase, and neuraminidase. It is inactivated after 15 minutes at 56°C, and 0.2% formaldehyde reduces the HA titer by eightfold. Apart from rat erythrocytes, most FAdV-1 strains do not agglutinate erythrocytes from other species. However FAdV-1 (Indiana C) was found to agglutinate sheep erythrocytes, suggesting some variation within serotypes. No evidence exists for hemagglutination by any of the other fowl serotypes or by the turkey or duck aviadenoviruses (18).

Strain Classification

Antigenicity

The adenovirus hexon is the major capsid protein and contains type, group, and subgroup specific antigenic determinants (97, 98). Birds infected with aviadenoviruses, therefore, produce type-specific, group-specific, and subgroup-specific antibodies. The group-specific determinants are shared by all subgroup I viruses, but are not present in subgroup II or subgroup III adenoviruses (although EDS virus appears to show some partial cross reactivity with subgroup I viruses, which has been demonstrated by indirect means). The double immunodiffusion (DID) test, which has often been used to demonstrate group-specific reactivity, can be used, therefore, to differentiate subgroup I, II, and III viruses.

Type-specific determinants give rise to antibodies that neutralize viral infectivity, and, therefore, the neutralization test has been widely used to separate isolates into serotypes (23, 39, 42, 67, 80, 81, 83, 99, 102). The current classification of subgroup I adenoviruses is shown in Table 9.2.

Immunogenicity and Protective Characteristics

Neutralizing antibodies produced against the type-specific epitopes should provide protection, but field and experimental experience indicates that the protection is relatively short lived.

Molecular

Restriction enzyme (RE) analysis was used to differentiate 12 recognized serotypes into 5 genotypes designated A–E (161), which are now recognized as separate adenovirus species (Table 9.2) (16). PCR has been applied to detection of subgroup I aviadenoviruses, with primer sequences based on the hexon gene (71) and allocation of viruses to species A–E and to serotypes within species is now frequently carried out by PCR (16, 71, 161).

Laboratory Host Systems

Most chicken isolates have been made in chick kidney (CK) or chicken embryo liver (CEL) cells. Although it has been claimed that CEL cells are more sensitive, there appears to be little difference when examining clinical material. However, CEL cells are preferable for diagnostic purposes because of their greater sensitivity to other viruses. Fowl adenoviruses form plaques in CK cells. Chicken tracheal organ cultures and chicken embryo fibroblasts appear to be less sensitive than other cells (98).

Adenoviruses have been isolated from turkeys and from a variety of other birds, including ducks (18), guinea fowl (120), pigeons, budgerigars, and mallard ducks (104, 152), using chicken cell cultures. Some turkey adenoviruses, however, grow only in turkey cells and do not grow or grow only poorly in chicken cells (123). It may be that if other avian species are examined using homologous cell systems, an extended range of viruses will be recognized.

Although it is probable that all aviadenoviruses multiply in the embryonated egg, not all chicken or turkey isolates cause recognizable lesions. The chorioallantoic membrane route of inoculation was found to be more sensitive for virus isolation than the allantoic cavity (80). High virus titers of all prototype strains except FAdV-3 (SR49) killed chicken embryos, but when low infectivity titers were used, only FAdV-1 (Ote) killed embryos. When material from naturally occurring adenovirus infections was used (20), only three isolations were made in embryonated eggs compared with 45 in cell culture. Most adenovirus isolates made in eggs have been typed as FAdV-1 or 5, which are not the most prevalent viruses in chicken populations when either serologic surveys (67) or virus isolation studies (40,41,157) have been carried out in cell cultures. However, inoculation into the yolk sac, and to a lesser degree onto the chorioallantoic membrane, supported the growth of 11 recognized serotypes (37). Signs and lesions produced in the embryo were death, stunting, curling, hepatitis, splenomegaly, congestion and hemorrhage of body parts, with urate accumulations in the kidneys. Hepatocytes usually contained basophilic or eosinophilic, intranuclear inclusion bodies. The FAdV-4 viruses associated with hydropericardium syndrome grew on the chorio-allantoic membrane and in the yolk sac on egg inoculation (6, 92).

Pathogenicity

Because the role of subgroup I adenoviruses as primary pathogens is not clearly established, factors determining pathogenicity are not clear. Different serotypes, and even strains of the same serotype, can vary in their ability to produce illness and death (19, 34) or respiratory disease (47). Variation in the ability to grow and persist in embryo tendon explants has also been noted (60). With some isolates, a relationship has been found between genotype and virulence but not between serotype and virulence (52). Although FAdV-1 produces a variety of tumors when inoculated into hamsters and will transform human and hamster cells (97), attempts to demonstrate oncogenicity with other avian serotypes have been unsuccessful (54).

In many studies, the route of inoculation has been extremely important in producing disease. Many isolates have failed to cause disease when inoculated by natural routes or by direct spread but were highly pathogenic when given by parenteral inoculation. This may suggest that many adenoviruses are potential pathogens but require the presence of some other agent to allow them to cause disease. Thus, coinfection with IBDV enhanced the pathogenicity of some aviadenoviruses (55,132), and the ability of some viruses to cause hepatitis and death was also considerably enhanced by coinfection with CIAV (19). In contrast, presence of an adenovirus-associated parvovirus may reduce the growth of the adenovirus in cell cultures as well as pathogenicity and oncogenicity (97).

Pathobiology and Epidemiology

Incidence and Distribution

Subgroup I avian adenoviruses are distributed widely throughout the world, and domestic avian species of all ages are susceptible. Other avian species appear to be susceptible to infection with fowl serotypes as well as serotypes of their own, but this has not been fully investigated.

Natural and Experimental Hosts

Chicken adenoviruses are ubiquitous in fowl populations, as demonstrated in antibody surveys (67, 157) and by the high levels of virus isolation from specimens taken from normal and sick birds (41, 80, 83, 101). In addition to infecting chickens, fowl adenovirus serotypes have been recovered from turkeys, pigeons, budgerigars, and a mallard duck (28, 104), and probable fowl isolates have come from guinea fowl (102) and pheasants (22).

Particles that are probably adenoviruses have been observed by electron microscopy in thin sections of tissue taken from kestrels (144), herring gulls (88), peach-faced lovebirds (153), a rose-ringed parakeet (45), budgerigars, rosella, red-rumped parakeets (109), eclectus parrots (124), common murre (90), a cockatiel (141), and a tawny frogmouth (127).

In addition to being infected with chicken serotypes, turkeys are also infected with adenoviruses that grow in cells of turkey origin but either do not grow or grow poorly in cells of fowl origin (140). Antibody to these viruses is widespread in turkeys.

Adenoviruses have been isolated from geese, and antibodies in geese are widespread. These viruses are unrelated in cross-neutralization tests to the recognized fowl serotypes but grow in cells of both goose and fowl origin (129, 160). A subgroup I adenovirus has been isolated from a Muscovy duck (18). This virus was unrelated to recognized fowl or turkey serotypes but did grow in chicken as well as duck cells. This virus is now classified as duck adenovirus type-2 (DAdV-2; Table 9.2), egg drop syndrome (EDS) virus being duck adenovirus type-1 (DadV-1).

Attempts to grow avian adenoviruses in mammals have met with very limited success. FAdV-1 has produced fibrosarcomas, hepatomas, ependymomas, and adenocarcinomas when injected into hamsters (97), and another isolate has produced hepatitis in hamsters (54).

Age of Host Commonly Affected

Some viruses cause mortality in day-old chicks, following inoculation, but not in 10-day-old birds (34). Virulence could be associated with the strain of virus, the age of bird, and the infectivity titer, with the minimum lethal dose ranging from 4 to >300,000 $TCID_{50}$ (15).

Transmission, Carriers, and Vectors

Vertical transmission is important in the spread of adenoviruses. Adenoviruses are transmitted through the embryonated egg and are often reactivated in cell cultures prepared from embryos and young chicks taken from infected flocks (98). This has been one of the strongest motivations for establishing SPF flocks. There is evidence that adenovirus infection can remain latent and undetected for at least one generation in an SPF flock (56).

Although adenoviruses can be isolated from day 1 following infection onward, viruses normally are excreted from week 3 onward. In broilers, peak excretion occurred between 4 and 6 weeks of age (97). In layer replacements, virus excretion was at a max-

imum level from 5–9 weeks following infection but was still at 70% after 14 weeks, and six adenovirus serotypes were isolated from samples taken from four farms (157). In a study beginning with 8-week-old birds (41), excretion continued at a high level until 14 weeks of age, and eight different serotypes were isolated from samples from seven farms. It is not uncommon to isolate two or even three serotypes from one bird, suggesting that there is little cross protection. Certainly birds can excrete one serotype in spite of high levels of neutralizing antibody to other serotypes. There appears to be a second period, around peak egg production, when adenoviruses are often detected. Presumably, the stress associated with egg production or the increased levels of sex hormones at this time caused reactivation of the virus. This would ensure maximum egg transmission to the next generation.

Horizontal spread of virus is also important. Virus is present in feces, the tracheal and nasal mucosa, and kidneys. Therefore, virus could be transmitted in all excretions, but highest titers are found in the feces. Virus may also be present in the semen, presenting a potential risk where artificial insemination is used. A juvenile as well as an adult pattern of virus excretion has been demonstrated. A 35-day-old bird showing the adult pattern had a lower peak titer of adenovirus present in the feces with an earlier decline in virus titer, and excretion persisted for a shorter time than in newly hatched chicks, which exhibited the juvenile excretion pattern (33). Horizontal spread within a flock seemed to take place mainly by direct fecal contact but was also achieved by aerial contact over short distances, with infection spreading at a very slow rate for several weeks (33). This pattern has also been seen in experimentally infected and in adventitiously infected SPF flocks and contrasts markedly with the normal pattern usually found in commercial flocks in which most birds in a flock are often excreting adenoviruses. In these circumstances, it is probable that many foci of infection exist, due to reactivation of latent viruses. Commercial flocks are often derived from a number of parent flocks, each of which harbor their own range of adenovirus serotypes. Consequently, cross infections resulting in the mixing of several serotypes take place when the birds are brought together. Aerial spread between farms does not appear to be important, except when cleaning of depopulated houses takes place, when the dust created can transmit infection between farms. Spread by fomites (e.g., egg trays and egg trolleys), personnel, and transport can also be important.

Incubation Period

Although adenoviruses have been associated with a number of clinical conditions, the evidence for a primary role in disease is conflicting. The incubation period is short (24–48 hours) following infection by natural routes.

Clinical Signs

Inclusion Body Hepatitis

Inclusion body hepatitis (IBH) is characterized by sudden onset of mortality peaking after 3–4 days and usually stopping on day 5 but occasionally continuing for 2–3 weeks. Morbidity is low; sick birds adopt a crouching position with ruffled feathers and die within 48 hours or recover (73,93,105). Mortality may reach 10% and occasionally as high as 30% (15). IBH normally is seen in meat-producing birds at 3–7 weeks of age, but it has been reported in birds as young as 7 days old (15) and as old as 20 weeks (76). There is evidence that in an integrated broiler operation, disease occurs in chickens from certain breeder flocks (93).

Many different serotypes have been associated with naturally occurring outbreaks of inclusion body hepatitis (IBH). Among those recorded are FAdV-1 (138); FAdV-2, FAdV-3, and FAdV-4 (65, 105); FAdV-5 (53, 104); FAdV-6, FAdV-7, and FAdV-8 (81); FAdV-7 and FAdV-10 (15); FAdV-8 (68 ,93, 105); FAdV-9 (69); and FAdV-12 (137).

Some workers have been successful in reproducing liver lesions with basophilic intranuclear inclusions (68, 95, 137) (see Fig. 9.4A), or both liver and pancreatic lesions (130) following parenteral inoculation of very young chicks with adenovirus. When 12-month-old birds were inoculated intravenously with an FAdV-1 virus (78), no clinical signs were seen, but there was degeneration, necrosis, and a cellular response in the liver, with a mild response in the trachea and lungs. Occasionally, liver lesions have developed following natural routes of infection in older birds (53), but more typically, no disease results even when unnatural routes of exposure are used (93,96).

Outbreaks of IBH in chickens less than 3 weeks of age with mortality up to 30% has occurred in Australia (15). IBH has been reproduced by inoculating day-old chicks by nasal and ocular routes with 24 isolates belonging to serotypes 6, 7, and 8. When these isolates were analyzed using restriction enzymes, it was found that all were FAdV-E viruses (52). In New Zealand, FAdV-8 was the most common isolate from IBH outbreaks, but FAdV-1 and 12 were also isolated. All belonged to the FAdV-E genotype (29) but differed from the Australian IBH FAdV-E isolates (137). These isolates produced focal hepatitis in 2-day-old birds infected orally. In contact, birds did not develop IBH but showed severe growth retardation (135).

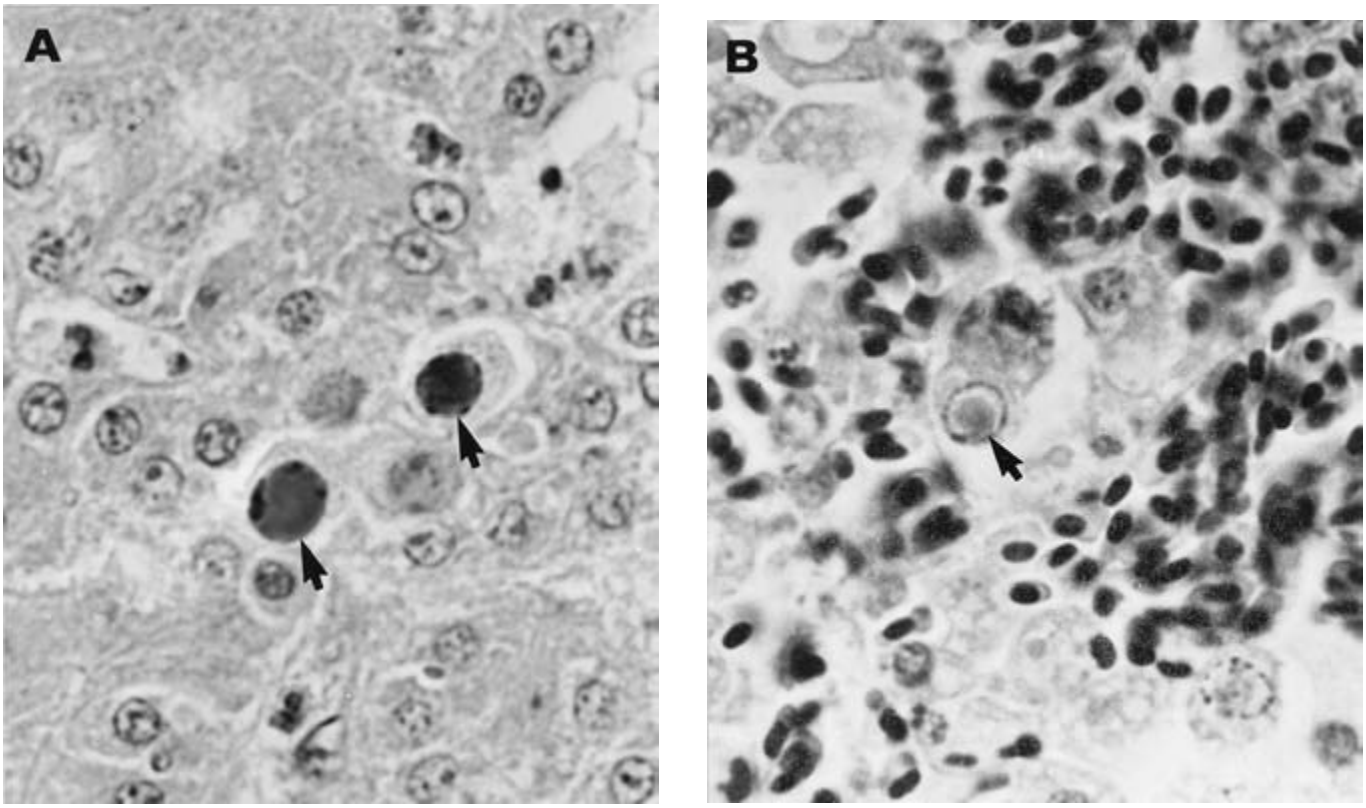
Immunosuppression induced by IBDV infection appears to facilitate adenoviruses in producing IBH (55, 132). In both Northern Ireland and New Zealand, however, IBH occurred in chickens before IBD was present in the country (29), and IBH has occurred spontaneously in SPF birds in the absence of IBD (126). When birds were infected with both CIAV and adenovirus, there was increased incidence of hepatitis and death (19).

Inclusion Body Hepatitis in Other Birds

A number of outbreaks of IBH have been recorded in pigeons. In addition to hepatitis, pancreatitis has also been found (35, 61, 82, 105, 146). Inclusion body hepatitis has been diagnosed in a group of eclectus parrots (124), kestrels (144), and a merlin (138), and an adenovirus was also recovered from day-old turkeys with IBH (64).

Hydropericardium Syndrome

In 1987, a new condition—Hydropericardium Syndrome or Angara disease—was recognized in Pakistan, and within one year it had devastated the national broiler industry in Pakistan. The disease subsequently was recognized in India, Kuwait, Iraq, Japan, and the former U.S.S.R. A very severe form of IBH was



9.4. A. Large basophilic intranuclear inclusion bodies in liver of a chicken experimentally infected with FAdV-8. B. Eosinophilic intranuclear inclusion body in hepatocyte of a chicken with naturally occurring inclusion body hepatitis.

diagnosed in South and Central America and Mexico. This disease was similar to hydropericardium syndrome, and it appears that the only differences between hydropericardium syndrome and IBH is that the mortality rate and incidence of hydropericardium is higher in the former disease. It caused between 20 and 80% mortality, with very low morbidity. Typically, mortality starts at 3 weeks, peaks for 4–8 days in weeks 4 and 5, and then declines (13, 38). Hydropericardium syndrome also occurs in breeding and laying flocks, with lower mortality rates. Adenoviruses are considered to be the cause of this condition, but some workers have claimed that other agents may also be involved (6, 13, 26). Twelve isolates from clinical outbreaks of infectious hydropericardium in 7 Asian and American countries were studied. Nearly all were shown to be FAdV-4 viruses, although one report included a FAdV-12 isolate, and there was also a genomic relationship between the FAdV-1 isolates (72, 121). Experimental studies indicate that there may be variation in virulence between strains (9). Some FAdV-4 isolates can reproduce the condition by themselves, and other strains appear to require the assistance of an immunosuppressive agent such as CIAV or exposure to immunosuppressive aflatoxins in the feed (143, 148).

The agent spreads well laterally among birds, and personnel appear to be important vectors (10, 11). Liver suspension from affected birds is infectious when inoculated by the oral or intranasal routes. Higher mortalities follow subcutaneous inoculation and the possibility that the initial massive spread throughout Pakistan was the result of a contaminated vaccine has been raised (10). In addition, this virus is also spread vertically (14, 150).

The disease has also been seen in pigeons (110). It was possible to reproduce the disease in broilers using liver suspensions from infected pigeons, and the disease in pigeons was controlled using the poultry vaccine.

Gizzard Erosions

There have been several reports over the last six years describing outbreaks of gizzard erosions in broilers from which a group I avian adenovirus has been associated (1, 118, 119). To date, these reports have originated in Japan, and have predominantly isolated a serotype 1 adenovirus, although one report has identified a serotype 8 adenovirus (113, 118, 119). No clinical signs except for mortality in young broilers have been reported in natural outbreaks. Gizzards are distended with hemorrhagic fluid, and contain multiple black patchy erosions within the koilin layer (1). A number of experimental inoculation studies using oral inoculation into young broilers or leghorns between 1 and 5 weeks of age reproduced the gizzard erosions in 3 to 18 days postinoculation (111, 113, 114, 116, 117). In some experiments, in addition to reproduction of gizzard erosions, investigators also found pancreatitis, hepatitis, cholecystitis, and cholangitis, indicating in-

fection spread through a number of digestive organs beyond the gizzard (113, 116).

Recently, a report from the United States described a similar condition of broilers to adenoviral-induced gizzard erosions, but this time the lesions were restricted to the proventriculus, and the authors termed this condition transmissible viral proventriculitis (TVP) (70). This adenovirus appears to be distinct from all known avian adenoviruses, failing to cross react with groups I, II, or III by IFA or PCR assays.

Aviadenovirus Infection in Chickens

Egg Production. Some workers have reported adenovirus infection resulting in a 10% fall in egg production (32) or an adverse effect on eggshell quality (40, 154). In a similar study, however, no effect on egg quality was demonstrated following experimental infection of birds with four strains of adenovirus, and only one virus strain had a minimal effect on the number of eggs produced (40). Adenoviruses can be isolated from commercial flocks, even when exceptionally high levels of production and fertility exist, and adenovirus infections of SPF flocks in lay are often associated with little or no effect on either egg production or shell quality.

Food Conversion and Growth. There have been reports of adenovirus infection resulting in decreased food consumption (40). Although birds inoculated with adenovirus may have depressed body weights and even high mortality (34, 66), there is little evidence to suggest that naturally occurring infection causes either reduced food utilization or growth. However, growth retardation did occur in naturally infected birds kept under experimental conditions (137). Chickens inoculated with adenovirus had reduced weight gain accompanied by excess fat deposition and depressed cholesterol and triglyceride levels (49). However, a study in Denmark was unable to detect any effect on broiler flock performance (77).

Respiratory Disease. Subgroup I aviadenoviruses frequently have been isolated from both the upper and lower respiratory tract of birds with respiratory disease (51, 79, 101, 115, 130). A survey of records of virus isolations, clinical, and necropsy findings over a 20-year period, involving hundreds of adenovirus isolations, indicated no primary role for adenoviruses in fowl respiratory disease. Before infectious bronchitis was controlled by vaccination and *Mycoplasma gallisepticum* and *Mycoplasma synoviae* were eradicated from poultry flocks, lesions in the respiratory tract were considerably more severe when adenoviruses were isolated from the respiratory tract specimens. Schmidt *et al.* (139) recorded similar findings and reported that mixed infectious bronchitis-mycoplasma-adenovirus infections presented with a similar clinical picture to infectious laryngotracheitis. Catarrhal tracheitis and multifocal pneumonic lesions, similar to lesions resulting from experimental adenovirus infections, were found in 13 outbreaks, leading to the conclusion that adenoviruses were a significant cause of respiratory disease (43).

Experimental infections have produced equivocal results. Using aerosol exposure, mild respiratory disease was produced (8). Following intratracheal inoculation, respiratory disease usually occurred (32, 96), but occasionally no signs were noted (42).

Tenosynovitis. Adenoviruses have been isolated from chickens with tenosynovitis, but experimental work has not confirmed their involvement (76).

Aviadenovirus Infection in Turkeys

Adenoviruses have been isolated from clinical outbreaks of respiratory disease, diarrhea, and depressed egg production in turkeys, but attempts to reproduce disease generally have been unsuccessful (51, 145).

Aviadenovirus Infection in Geese and Ducks

Three serotypes isolated from geese did not reproduce disease in goslings (160). In outbreaks of high mortality associated with hepatitis, adenovirus-like particles were seen in the liver (128).

A diphtheroid, stenosing tracheitis with occasional bronchitis and pneumonia was seen in up to 10% of 7–21-day-old Muscovy ducks, and the tracheal epithelial cells contained numerous adenoviruses (17). A similar condition was recently described in geese in Canada (129), where an isolated parent flock became infected with adenovirus, and produced two hatches in which mortality reached 12% in 4–11-day-old goslings.

Aviadenovirus Infection in Pigeons

Adenoviruses have been associated with two clinical diseases in pigeons in Belgium, described as adenovirus type I (classical adenovirus), and type II (necrotising hepatitis) (44, 82). Classical adenovirus occurs in pigeons under 1 year of age and appears to be associated with the stress associated with the first pigeon races. The virus replicates in the nuclei of epithelial cells of the intestinal tract and also in the liver. The affected pigeons have watery diarrhea, vomiting, and weight loss. There is rapid spread within affected pigeon lofts, with up to 100% morbidity in birds under 1 year old within 2 days. Uncomplicated cases resolve in about 1 week, but secondary bacterial infections can lead to more severe and prolonged disease and death. Pigeons that recover suffer greatly impaired racing performance.

Necrotising hepatitis occurs throughout the year in pigeons of all ages. There are often only minimal signs of disease, however, vomiting and yellow liquid droppings have been reported in some cases, although sudden death within 24–48 hours of infection is often the only sign. The virus induces massive hepatic necrosis in affected birds. New cases in a pigeon loft may occur over a period of 6 weeks, with total mortality reaching 30%, although 100% mortality has been reported. The birds that do not die remain completely normal. On autopsy, the liver is yellow in color and swollen. There is extensive hepatic necrosis, with eosinophilic or amphophilic intranuclear inclusion bodies in hepatocytes (44). These conditions have been reviewed by Vereecken *et al.* (152).

Aviadenovirus Infection in Guinea Fowl

Two strains of adenovirus were isolated from naturally occurring outbreaks of pancreatitis in guinea fowl. One strain induced se-

vere disease and death with respiratory and pancreatic lesions after oral and nasal infection of 1-day-old guinea fowl (120). Foci of necrosis with large basophilic and smaller eosinophilic inclusion bodies were seen in guinea fowl suffering from necrotic pancreatitis (125). An FAdV-1 isolate from guinea fowl with necrotizing pancreatitis reproduced the condition experimentally (85). A hemorrhagic disease of guinea fowl in which adenoviral inclusions were present in the spleen has been described and reproduced experimentally (91).

Aviadenovirus Infection in Ostriches

Adenoviruses have been associated with illness, death, and poor hatchability on ostrich farms (122), and an isolate from an ostrich caused pancreatitis in guinea fowl (24, 63). In a study in which 3-day-old ostrich chicks were inoculated with an ostrich-derived adenovirus, all died (123).

Pathology

Inclusion Body Hepatitis

In the literature are several descriptions of a disease primarily affecting the liver and also one in which the primary lesions appeared to be present in the hemopoietic system. It is probable that the aplastic anemia described was due to infection with CIAV (159) (see Chapter 8). The main lesions of IBH are pale, friable, swollen livers. Petechial or ecchymotic hemorrhages may be present in the liver and skeletal muscles (73, 93, 105).

Inclusion bodies are seen in the hepatocytes. These can be eosinophilic, large, round, or irregularly shaped with a clear pale halo (67, 68, 74) or occasionally basophilic (67, 68, 74) (Fig. 9.4A, B). In IBH cases in Australia, basophilic inclusions appear to predominate (15, 81). Virus particles were detected only in cells with basophilic inclusions, and the eosinophilic inclusions were composed of a fibrillar, granular material (75). In New Zealand, lesions included atrophy of the bursa and thymus, aplastic bone marrow, and hepatitis. Inclusions were eosinophilic. The description of bursal and thymic atrophy in the absence of IBD is interesting (29).

Hydropericardium Syndrome

In cases of hydropericardium syndrome, there is an accumulation of clear straw-colored fluid in the pericardial sac, pulmonary edema, swollen and discolored liver, and enlarged kidneys with distended tubules showing degenerative changes. Multiple areas of focal necrosis exist with mononuclear infiltration in the heart and liver. Basophilic inclusions are present in the hepatocytes (13). One report describes multifocal pancreatic necrosis and gizzard erosions in associated with hydropericardium syndrome (112).

Respiratory Disease

In naturally occurring outbreaks, mild to moderate catarrhal tracheitis with excess mucus were the only gross lesions noted (46). Hyperemia of the lungs, cloudy air sacs, and petechial hemorrhages in the pharynx and larynx were described after experimental infection (7). Microscopically, the main lesions were loss of cilia, necrosis of some epithelial cells, intranuclear inclusion bodies, and infiltration of mononuclear cells into the lamina propria. Multifocal or occasionally diffuse interstitial pneumonia was found (7, 46, 47). Following aerosol exposure, epithelial hyperplasia, edema, and infiltration by mononuclear cells were seen in the air sacs (7).

Necrotizing Pancreatitis and Gizzard Erosions

Focal pancreatitis and gizzard erosions have been described in broiler chickens in both natural outbreaks and experimental studies in Japan (113, 116). Intranuclear inclusion bodies containing adenovirus antigen in glandular epithelial cells were associated with necrosis of the koilin layer, and infiltration of the lamina propria, submucosa and muscle layers by macrophages and lymphocytes (1, 111, 112, 116, 117, 118, 119). Intranuclear inclusions have also been demonstrated in necrotic pancreatic acinar cells (112, 116, 147). Pancreatitis associated with adenoviral infection has been recorded in guinea fowl (120, 125).

Immunity

Subgroup I avian adenoviruses have a common group-specific antigen that is distinct from that of human adenoviruses (24, 80, 103). There are differences in the degree to which this antigen is shared between serotypes. For example, FAdV-1 gave a strong reaction with its own antiserum, but FAdV-1 antigen failed to detect antibody to FAdV-2 or FAdV-4 (80). A microtiter fluorescent antibody test (4) confirmed these differences in titer.

Subgroup I adenoviruses isolated from turkeys (TAdV-1 and 2; Table 9.1) have a common antigen detected in double immunodiffusion (DID) tests, and this distinguishes them from the subgroup II (turkey hemorrhagic enteritis) adenoviruses (50, 103). The adenovirus isolate from a Muscovy duck (DAdV-2) was also reported to share an antigen with FAdV-1 (18).

Following infection, birds rapidly developed neutralizing (type-specific) antibodies that were detectable after 1 week and reached peak titers after 3 weeks (158). Group-specific antibodies were also detected by DID following infection but were often transient. Their detection probably depends on the sensitivity of the test used (32, 103, 158). Antibodies have also been detected using indirect immunofluorescence (4).

The development of neutralizing antibodies coincides with cessation of virus excretion. Young chicks excrete virus longer because of slower development of neutralizing antibody (31).

It has been found that birds were resistant to reinfection with the same serotype 45 days after primary infection (30). In another study (158), however, birds were reinfected with the same strain after 8 weeks, eliciting a secondary response of both neutralizing and precipitating antibodies. Virus excretion also occurred, despite the presence of humoral antibodies. Peaks of virus excretion were found when birds were 2.5, 4.5, and 7.5 months of age (84), consistent with the theory that local immunity lasts about 8 weeks but then regresses allowing virus to replicate again at mucosal surfaces.

Neutralizing antibodies induced by an inactivated vaccine had no effect on virus excretion in the feces but did reduce pharyngeal excretion, possibly by preventing hematogenous spread of virus from the intestine to the pharynx. It is, therefore, possible that the resistance to challenge found after infection is due to

short-lived local immunity while circulating antibodies protect mainly against invasion of the internal organs. The apparent correlation between appearance of circulating antibodies and cessation of virus excretion is more likely due to concurrent development of both local immunity, which is more transient, and humoral immunity, which is more persistent. Support for this hypothesis is provided by the finding that maternal antibody does not protect against natural routes of infection (30) but does protect against intra-abdominal infection (53,66). There is evidence that infection with some strains of aviadenovirus results in severe depletion of lymphocytes in the bursa, thymus, and spleen, causing immunosuppression (136).

Diagnosis

Isolation and Identification of Adenoviruses

Specimens of choice for virus isolation are feces, pharynx, kidney, and affected organs (e.g., liver, in cases of IBH). A 10% suspension of tissue in medium is prepared and, in the case of chickens, inoculated onto either chick embryo liver cells or chick kidney cells. Chick embryo fibroblasts and tracheal organ cultures are less sensitive. Although three passages have been used (20, 41), generally two blind passages of 7 days' duration each are sufficient. If attempting to isolate adenoviruses from other avian species, it is preferable to use cells from the species being investigated, although chicken cells can be used. However, some turkey adenoviruses either grow poorly or not at all in chick cell cultures (140). Embryonated eggs are insensitive for primary isolation of most aviadenoviruses, although Cowen (37) has shown that the yolk sac is a sensitive route for isolating laboratory strains representative of 11 serotypes. It remains to be seen whether this route of embryo inoculation is equally sensitive for isolation of adenoviruses from field material, but it clearly should be tried in laboratories lacking cell culture technology.

Confirmation of a virus isolate as an adenovirus can be carried out in a number of ways. Direct examination of the lysate from an infected cell culture by electron microscopy gives a quick and positive answer and has the added advantage that parvoviruses can also be detected if present. Immunocytochemistry can be used to detect adenoviruses in infected cells by staining with an avian adenovirus antiserum labeled with a fluorescent dye, such as fluorescein isothiocyanate (direct immunofluorescence) (Fig. 9.2). To identify the serotype of virus that has been isolated, virus neutralization tests with the isolate against standard reference antiserums to all known serotypes (23, 24, 39, 99, 102) has been widely applied. Confirmation using molecular methods is also available and is now widely used. Detection of adenovirus DNA in tissue samples by *in situ* hybridization (ISH) has been reported (71), and ISH probes based on reported sequences of FAdV-10 and FAdV-1 have been used (62, 86). PCR has been widely applied to detect subgroup 1 adenoviruses and can be used to allocate isolates to species and to serotype. The rationale for the design of primers is based on a number of considerations, in particular the purpose for which the test is required. Thus, while a general diagnostic PCR that detects adenoviruses from subgroups I, II and III is possible (71), primers that detect individual species or serotypes are generally more useful. Studies with FAdV-4 strains isolated from cases of infectious hydropericardium syndrome in different countries and locations have shown the usefulness of this approach (58, 72, 151). If these techniques are not available, then H&E staining of infected cell monolayers, or tissue sections, and demonstration of intranuclear basophilic inclusions, provides a nonspecific indication of the presence of DNA containing virus (Fig. 9.3).

Serology

Antibody to the group-specific antigen can be detected using the double immunodiffusion (DID) test, but its apparent sensitivity in naturally occurring outbreaks is often due to multiple infection with adenovirus serotypes, making it unreliable for detecting infection in SPF flocks (32, 56, 103). However, use of a trivalent antigen incorporating three adenovirus serotypes increases sensitivity of the DID test (36). The indirect immunofluorescent test is much more sensitive and rapid and is inexpensive (4), but its interpretation requires some previous training and experience. The enzyme-linked immunosorbent assay (ELISA) has been used to detect group-specific antibodies, and it is inexpensive and sensitive (24, 43, 107). Type-specific antibodies normally have been detected using the serum-neutralization test, but ELISA can also be used for this purpose (107).

The main problem with any serologic test for adenoviruses is the interpretation of the results. Adenovirus-specific antibodies are common in both healthy and diseased birds, and birds are frequently infected with a number of serotypes. Virus genotype may be of more interest than serotype for predicting disease-producing capability. Furthermore, the presence of humoral antibody gives no indication of the state of local immunity at mucosal surfaces.

Intervention Strategies

Management Procedures

As both IBDV and CIAV can potentiate the pathogenicity of adenoviruses, the first step must be to control or eliminate these two viruses. Adenoviruses are resistant to inactivation, and although it is possible to eliminate them from most environmentally controlled houses with impervious floors and walls that can be made airtight, the value of attempted eradication of adenovirus from commercial flocks is questionable. This is because the virus is effectively transmitted vertically through the embryonated egg, so that adenoviruses would almost certainly be introduced to subsequent flocks. Therefore, effective control would have to start at primary breeder level. Furthermore, experience with SPF flocks has indicated that horizontal spread is also a major problem, and it would be exceedingly difficult to keep a commercial flock free from adenovirus infection.

Vaccination

As evidence mounts that certain genotypes may be primary pathogens, the possibility of vaccination is more appealing. Maternal antibody titers of 1/64 or greater were successful in preventing the development of IBH, but the birds experienced some growth retardation (156). A vaccine prepared by inactivating

homogenates of liver from infected birds has been used extensively with apparent success in Pakistan to control hydropericardium syndrome (5, 12, 133). One study has shown enhanced protection against hydropericardium syndrome by vaccinating broiler breeders against both CIAV and FAdV-4 followed by experimental challenge of their progeny, compared to lesser protection achieved by vaccinating against either disease alone (149). This reemphasizes the importance of management to control immunosuppressive viral infections in order to decrease the effects of aviadenoviruses. The genome of FAdV-1 (CELO) has been shown to have substantial capacity for insertion of foreign DNA sequences, and consequently there is substantial interest in the development of CELO as a vaccine vector for use in avian species (106). Experiments in which infectious bursal disease virus (IBDV) sequences were incorporated demonstrated efficient expression and processing of IBDV proteins indicating that the CELO virus offers potential as a vaccine delivery vehicle or as a vector for production of recombinant proteins (57).

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Egg Drop Syndrome

Brian McConnell Adair and Joan A. Smyth

Introduction

Definition and Synonyms

The egg drop syndrome (EDS) virus has recently been reclassified (12). It was originally designated as the sole member of the subgroup III avian adenoviruses, but it has now been moved to a new genus, the genus *Atadenovirus* (Table 9.1). Ovine adenovirus D is the type species of this genus, and EDS virus is currently the only recognized atadenovirus from avian species. It is serologically unrelated to the aviadenoviruses (formerly called subgroup I), and the siadenoviruses (formerly called subgroup II). Only one serotype is recognized, although variations have been demonstrated in restriction endonuclease analysis of isolates (53,58).

Since its initial description (64), EDS virus has become a major cause of lost egg production throughout the world. It is caused by an adenovirus, probably introduced into chickens through a contaminated vaccine. The disease is characterized by the production of thin-shelled or shell-less eggs by otherwise healthy birds. Once established in a breeding organization, the condition more often is seen as a failure to achieve production targets, and eggshell changes are less apparent. Since its initial recognition, it has become apparent that sporadic outbreaks of EDS occur as a result of fowl becoming infected through direct or indirect contact with infected wild or domestic waterfowl.

History

A condition of laying hens was described by Dutch workers in 1976 (64), and hemagglutinating adenoviruses were isolated (42). Through serologic studies with one of these isolates and examination of flock records, it was possible to establish the disease pattern (42, 44). The virus appeared to be transmitted vertically through the egg, and horizontal transmission between flocks was not a feature of the disease. The virus often remained latent until birds were approaching peak egg production. Because of the absence of antibody to the virus in chickens prior to 1974 and the failure of the virus to grow in mammalian cells, as well as its poor growth in turkey cells and optimal growth in duck cells, it was suggested that this was probably a duck adenovirus. This suggestion was quickly confirmed by isolation of EDS virus from normal ducks and demonstration of antibody in many duck flocks (9, 16).

Public Health Significance

The virus affects only avian species and, therefore, has no public health significance.

Etiology

Classification

EDS virus is classified as an adenovirus on the basis of its morphology, replication, and chemical composition. The virus is not related to 11 fowl and 2 turkey prototype aviadenoviruses using serum neutralization (SN) or hemagglutination inhibition (HI) tests (3). Although the aviadenovirus group specific antigen is not detected by immunodiffusion or immunofluorescence tests in EDS virus preparations, presence of common antigenic determinants were indicated in experiments in which aviadenovirus group-specific antibodies, which had developed in chickens after inoculation with an aviadenovirus, were restimulated following a subsequent inoculation with EDS virus (43).

Sequencing of the genome of EDS virus indicated some significant differences from the subgroup I aviadenoviruses (28). These differences include smaller genome size (33.2kb compared to 43.8kb for FAdV-1) and high AT content (28). Also although some aviadenovirus early genes appear to be missing in EDS virus, other genes were identified that have no obvious homology with known aviadenovirus proteins (28). EDS virus was shown to have similarities in its genetic characteristics to an ovine adenovirus (strain 287), certain bovine adenoviruses, and a possum adenovirus. This group is sufficiently different from the mammalian adenoviruses (mastadenoviruses), and from the aviadenoviruses (subgroup I) and siadenoviruses (subgroup II) to warrant classification as a separate adenovirus genus, for which the name *Atadenovirus* has been proposed, to reflect the high AT content of the viral DNA (11,12,13). Although initial isolates of EDS virus were from chickens (44), the virus is now recognized

as having originated from ducks, and its species designation is as duck adenovirus type A, and its strain name as duck adenovirus type 1 (DAdV-1), or egg drop syndrome virus (12). A study of recent Japanese isolates from chickens showed no evidence of change of the virus after two decades of the virus circulating in chickens (63).

Morphology

Ultrastructure

Purified virus preparations from cesium chloride (CsCl) gradients showed typical adenovirus morphology with triangular faces having six capsomeres on the edge and a single 25-nm fiber projecting from each vertex (35). In nonpurified preparations, the detail of the surface structure is not obvious (44, 69). Although EDS virus particles are clearly adenoviruses with well-defined capsomeres with hollow centers, it is possible to distinguish them from conventional adenoviruses in some electron microscope preparations (Fig. 9.5). In thin sections of infected chick embryo liver cells, virus particles of 70–75 nm are seen in the nucleus (3). Particles of 68–80 nm in diameter have also been described in the nuclei of epithelial cells of oviduct mucosa (60). The EDS virus has a single fiber per penton base, unlike the aviadenoviruses, which have two fibers (35).

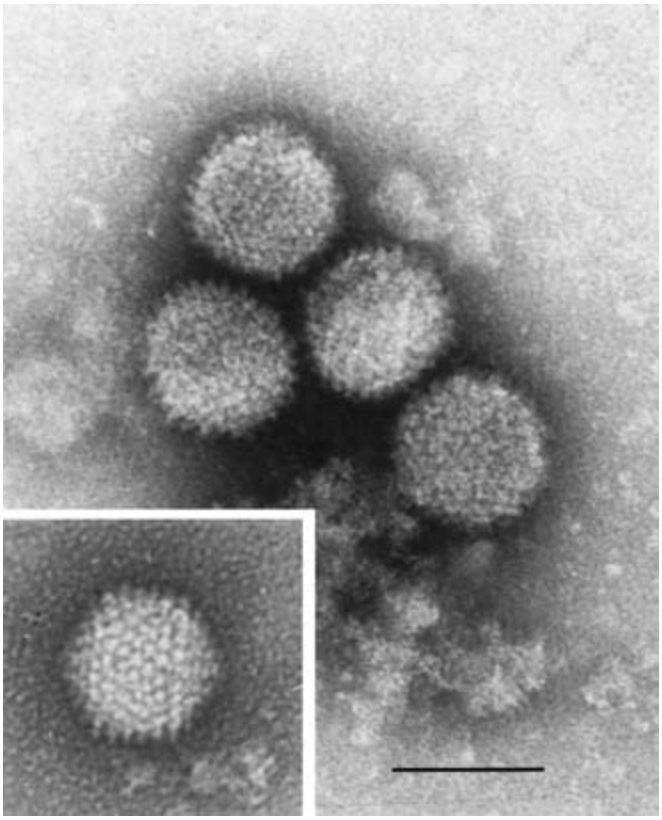
Size and Density

The size of EDS virus observed in negatively stained preparations has been reported to range from 76 nm (44) to 80 (65) nm (35). These sizes are within those acceptable for adenoviruses (68).

Differences in the reported density of EDS virus in CsCl have been reported. Todd and McNulty (61) found that infectious virus particles banded at densities of 1.32 and 1.30 g/mL. The heavier particles, however, did not agglutinate chicken erythrocytes and appeared by electron microscopy to be slightly damaged. Particles with a density of 1.30 g/mL hemagglutinated and appeared undamaged. A band of empty, disrupted, noninfectious hemagglutinating particles was present at a density of 1.28 g/mL. In contrast, Kraft *et al.* (35) reported presence of two bands of infectious, hemagglutinating particles at 1.32 g/mL, with noninfectious disrupted particles banding at 1.30 g/mL. Yamaguchi *et al.* (69) reported hemagglutinating particles banding at 1.30 g/mL and infectious particles with a density of 1.33 g/mL, and Takai *et al.* (59) found infectivity and hemagglutinin associated with a band at 1.33 g/mL and hemagglutinin in a band at 1.29. This discrepancy was explained, at least in part, by Zsak and Kisary (74), who reported that density and hemagglutinating ability of EDS virus particles depended on the method used for virus purification and whether the virus was grown in cell cultures or embryonated eggs.

Chemical Composition

Labelling with H^3-thymidine and inhibition with iododeoxyuridine showed that EDS virus contained DNA (3, 35, 61, 69). The molecular weight of the DNA was estimated at 22.6×10^6 d compared with 28.9×10^6 d for FAdV-1 (Phelps), and restriction endonuclease patterns indicated no relationship between these two viruses (74). The EDS virus has 13 structural polypeptides, at least 7 of which correspond with polypeptides of FAdV-1 (61,71).



9.5. Four particles of egg drop syndrome (EDS) virus. Although individual capsomeres are well resolved, typical adenovirus morphology is not apparent. Inset: Fowl adenovirus type 8 (FAdV-8) particle showing well-defined, triangular faces. Bar = nm.

Hemagglutination

EDS virus agglutinated erythrocytes of chickens, ducks, turkeys, geese, pigeons, and peacocks but did not agglutinate rat, rabbit, horse, sheep, cattle, goat, or pig erythrocytes (3, 38).

The hemagglutinin (HA) is resistant to heating at 56°C. An initial fourfold fall in HA titer was reported after 16 hours at 56°C, and thereafter the titer remained stable for 4 days; however, no HA activity was detected after 8 days at 56°C. The HA also survived heating at 60°C but was destroyed by heating to 70°C for 30 minutes. The HA activity was also stable for long periods at 4°C (3,45) and was resistant to treatment with trypsin, 2-mercaptoethanol, ethylenediaminetetraacetic acid (EDTA), papain, ficin, and 0.5% formaldehyde at 37°C for 1 hour, but the titer was greatly reduced by treatment with potassium periodate and 0.5% glutaraldehyde (59). However, the purified soluble HA was destroyed by trypsin treatment (61). Alpha-chymotrypsin destroyed the virus receptor on chicken erythrocytes; whereas trypsin and neuraminidase had no effect (59).

Virus Replication

EDS virus replicates in the nucleus of infected cells in a similar fashion to aviadenoviruses (1, 2, 3). Intranuclear inclusions were observed in hematoxylin- and eosin-stained preparations of virus-infected cell cultures (3), in epithelial cells of the infundibulum, tubular shell gland, pouch shell gland, isthmus, and in nasal mucosa and spleen of experimentally infected chickens (57,60). In ultrathin sections of EDS virus-infected cells examined by electron microscopy, adenovirus particles and type I–IV inclusions similar to other avian adenoviruses were obvious in the nucleus (2, 3).

Susceptibility to Chemical and Physical Agents

The EDS virus was stable to treatment with chloroform and variations in pH between 3 and 10. The virus was inactivated by heating for 30 minutes at 60°C, survived for 3 hours at 56°C, and was stable in the presence of monovalent but not divalent cations (3, 69). Infectivity was not demonstrated after treating with 0.5% formaldehyde or 0.5% glutaraldehyde (59).

Strain Classification

Only one serotype of EDS virus has been recognized (21,69). With the use of restriction endonuclease analysis, however, it has been possible to divide a number of isolates into three genotypes (62). One group included isolates made over an 11-year period from infected European chickens, and a second group included viruses isolated from ducks in the United Kingdom. One virus isolated from chickens in Australia formed the third group.

Laboratory Host Systems

EDS virus replicated to high titers in duck kidney, duck embryo liver, and duck embryo fibroblast cell cultures and grew well in chick embryo liver cells, less well in chick kidney cells, and rather poorly in chicken embryo fibroblast cells. Growth in turkey cells was poor, and no replication was detected in a range of mammalian cell cultures (3). The virus grew to high titers in goose cell cultures (74). In chick liver cell cultures, peak virus and intracellular HA titers were reached after 48 hours, with peak extracellular HA titers being achieved after 72 hours (69). The virus also grew very well when inoculated into the allantoic sac of embryonated duck or goose eggs producing HA titers of 1/16,000–1/32,000. No growth was detected in embryonated chicken eggs (3, 71).

Pathogenicity

Although all chicken isolates of EDS virus appeared to be of similar virulence, isolates from ducks in the United States produced no effect on egg production in chickens (65) or affected only the egg size (15). Isolates from ducks and chickens in Europe behaved in an identical manner in chickens (7).

Pathobiology and Epidemiology

Incidence and Distribution

EDS virus has been isolated from chickens in Australia (24), Belgium (45), China (73), France (48), Great Britain (9), Hungary (74), India (36), Israel (40), Italy (72), Japan (69), Northern Ireland (44), Singapore (54), South Africa (14), and Taiwan (38). Serologic evidence of infection has been found in chickens in Brazil (31), Denmark (5), Mexico (51), New Zealand (30), and Nigeria (46).

Natural and Experimental Hosts

Although disease outbreaks have been recorded mostly in laying hens, it is probable that the natural hosts for EDS virus are ducks and geese. EDS virus HI antibody is widespread in domesticated ducks (5, 8, 9, 16, 24, 39, 40) and domestic geese (8, 75). In a study of ducks in the Atlantic flyway in the United States, antibody was found in ruddy, ring-necked, wood, bufflehead, lesser scaup, mallard, northern shoveler, and gadwall ducks and in mergansers, coots, and grebes (27,51). Antibody was also detected in Muscovy ducks and in cattle egrets (40), Canada geese (51), herring gulls (8), owls, a stork, and a swan (33).

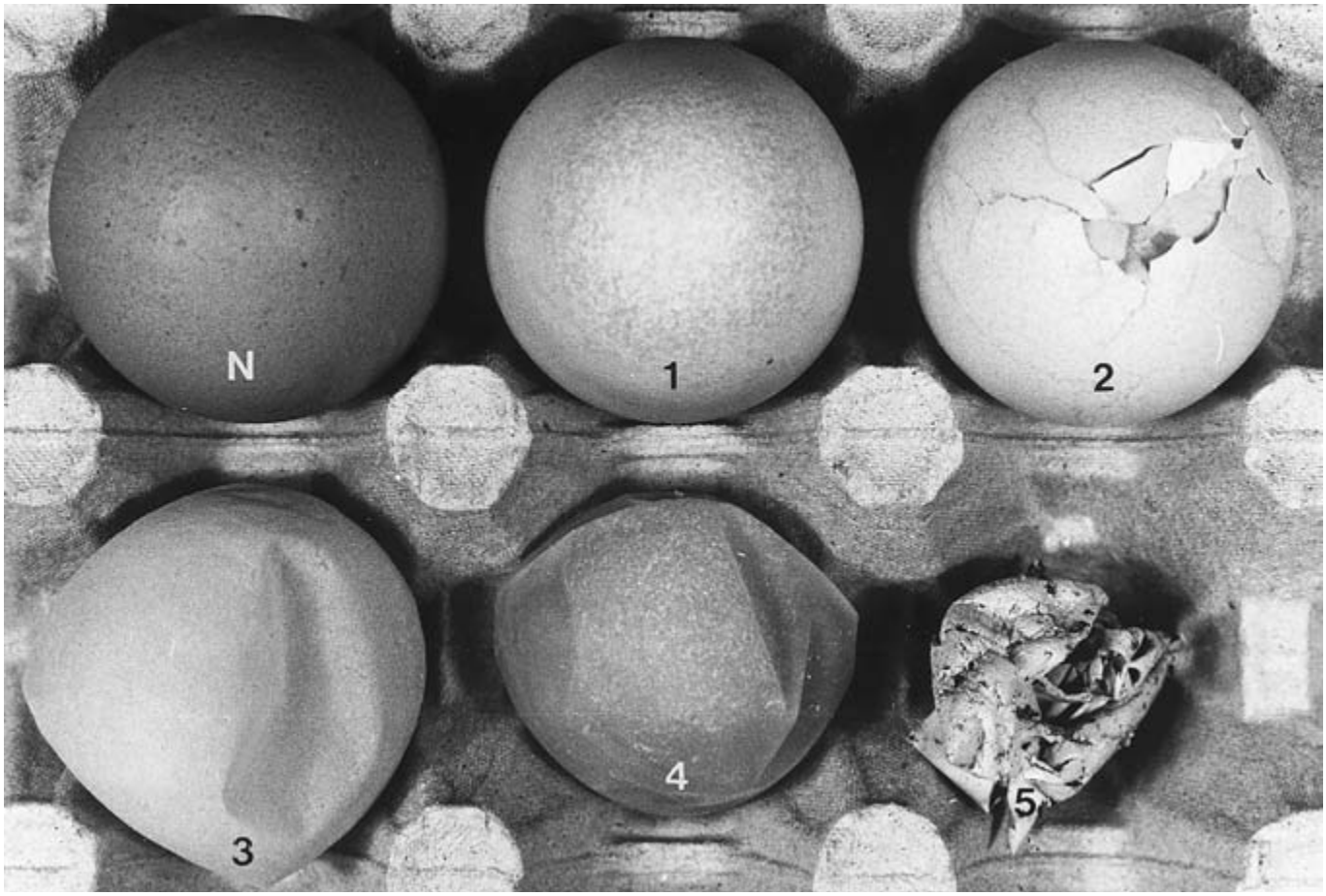
The virus has been isolated from healthy domestic ducks (9, 65) and from diseased ducks (25), but the disease could not be reproduced using this isolate. A virus was isolated from ducks with a fall in egg production and severe diarrhea (6), and it has been suggested that EDS virus may cause rough, thin shells and decreased egg production in ducks (23,37). EDS virus was isolated from the latter ducks, but experimental reproduction was not attempted. It remains possible that the viruses recovered from these ducks were incidental, as the virus can be found commonly in healthy ducks.

Infection was also shown to be common in geese (33,39,75), but goslings and geese experimentally infected with the virus showed neither illness nor change in egg production (75). However, there has been a recent report of a severe respiratory disease outbreak in young goslings, in which adenoviral inclusion bodies were found in the trachea and bronchial epithelium of affected birds. EDS virus was recovered from affected geese and the disease was reproduced experimentally in 1-day-old goslings (32).

Quail (*Coturnix coturnix japonica*) have also been shown to be susceptible to infection and to develop classic signs of EDS (22). There is no evidence of naturally occurring infection of turkeys or pheasants, although they can be infected experimentally (8,47,72). Guinea fowl may be infected naturally or experimentally, and soft-shelled eggs may be produced. However, in other studies, guinea fowl became infected but remained clinically normal following exposure to a fowl isolate of EDS virus (66).

Because EDS virus initially was transmitted vertically in chickens, there was often an apparent association with certain chicken breeds. A wide range of breeds, however, have been shown to be equally susceptible to experimental infection, although analysis of naturally occurring outbreaks suggests that broiler breeders and heavy breeds producing brown eggs are more severely affected than white-egg producers. When two brown and one white-egg layer strains were infected (29), egg production was depressed in the white layer strain. There was little depression in egg production of the brown layers, but more eggs were produced with shell defects. One brown egg-laying strain produced almost three times as many affected eggs as the white egg-laying strain.

Chickens of all ages are susceptible to EDS virus infection. Introduction of EDS virus into a laying site affected egg production in all ages of laying hens. However, the appearance of disease at around peak egg production (44) may be due to reactivation of latent virus.



9.6. Eggs from hens infected with egg drop syndrome (EDS) virus. Changes range from normal brown egg (N), to loss of shell pigment (1 and 2), thinning at the pole (1), thin-shelled (2), soft-shelled (3), and shell-less eggs (4). Eggs may be eaten or broken, but many membranes (5) may be found.

Transmission, Carriers, and Vectors

It is now possible to divide EDS outbreaks into three types. In the initially observed classic form of the disease, primary breeders were infected, and the main method of spread was vertically through the embryonated egg (44). Although the number of infected embryos was probably low with this type (10), lateral spread of virus was very efficient. In many cases, chicks infected *in ovo* did not excrete virus or develop HI antibody until the flock had achieved greater than 50% egg production. At this stage, the virus was reactivated and excreted, resulting in rapid spread due to multiple foci of infection.

Probably arising from the classic form of EDS, the virus has become established in commercial egg-laying flocks in some areas. In India, 32.6% of poultry flocks were found to be infected (36). This endemic form was often associated with a common egg-packing station. Both normal- and abnormal-shelled eggs produced during the period of virus growth in the pouch shell gland contained virus on their exterior and interior (56). This led to contamination of egg trays. Droppings also contained virus, but this excretion was intermittent, and the virus was often present only at low titer (18). In the adult bird, presence of virus in the feces probably arises from contamination by oviduct exudate (57). Apart from direct spread between birds, there is evidence that spread can occur when birds are transported in inadequately cleaned trucks or when unused food has been moved between sites. There is also evidence that needles or blades used for vaccination or bleeding of viremic birds, if not properly sterilized, can transmit infection. Lateral spread of virus is slow and intermittent, taking up to 11 weeks to spread through a cage house, and in one case, spread to an adjoining pen was prevented by a wire fence. Spread of virus between birds on litter is usually faster (18,64).

Spread of EDS virus from domestic or wild ducks, geese, and possibly other wild birds to hens through drinking water contaminated by droppings appears to give rise to a third type of disease outbreak. This type of disease is very important in some areas, and cases tend to be sporadic, but there is always the danger of an infected flock becoming the focus for endemic infection.

Clinical Signs

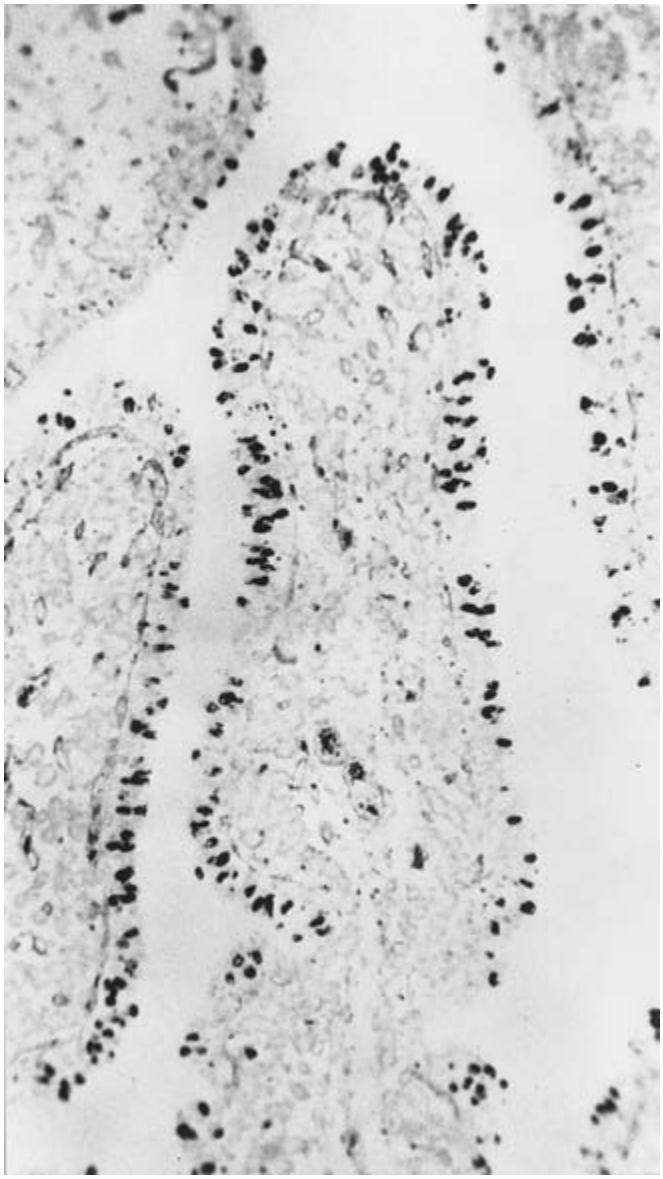
Following experimental infection, most workers detected the first signs of EDS after 7–9 days (19,41), but in some experiments, disease signs did not appear until 17 days PI (45).

The first sign was loss of color in pigmented eggs. This was quickly followed by production of thin-shelled, soft-shelled, or shell-less eggs (Fig. 9.6). Thin-shelled eggs were often rough,

with a sandpaper-like texture or had a granular roughening of the shell at one end of the egg. If the abnormal eggs were discarded, there was no effect on fertility or hatchability and no long-term effect on egg quality. If birds were infected in the late stages of egg production, forced molting of the flock appeared to restore egg production to normal. The fall in egg production was very rapid or extended over several weeks. EDS outbreaks usually lasted 4–10 weeks, and egg production was reduced by up to 40%; however, there was often compensation later in lay, so that the total number of eggs lost was typically 10–16/bird. If the disease resulted from reactivation of latent virus, the fall usually occurred when production was between 50% and peak level. Small eggs have been described in naturally occurring outbreaks (44), but no effect on egg size was found in experimental infections (41). Watery albumen has been described (45,64), although no effect on albumen was reported by other workers (21,41,70). Age at time of infection may be important, however; birds infected at 1 day of age produced apparently normal eggs except for impaired albumen quality and smaller size (19).

If some birds have acquired antibody before latent virus is reactivated, an apparently different clinical syndrome is seen. There may be failure to achieve predicted egg production, and onset of lay may be delayed. If a careful examination is made, it can often be established that there have been a series of small clinical episodes of classic EDS. Presumably, presence of EDS-specific antibodies slows down the spread of the virus in the bird. A similar picture has often been observed in birds housed in caged units, where spread of the virus can be slow and EDS not suspected.

Affected birds remain otherwise healthy. Although inappetence and dullness have been described in some affected flocks, these are not consistent findings. Transient diarrhea described by some authors is probably due to the exudate from the oviduct (57). EDS virus does not cause clinical disease in growing chickens in the field. Oral infection of susceptible day-old chicks resulted in increased mortality in the first week of life (19), but there was no increase in mortality in many flocks of chickens produced by virus-infected parent flocks.



9.7. Pouch shell gland from a hen experimentally infected with egg drop syndrome (EDS) virus. Note the viral nucleic acid in the surface epithelial layer, demonstrated by a biotinylated purified virus genome probe. (Allan)

Pathology

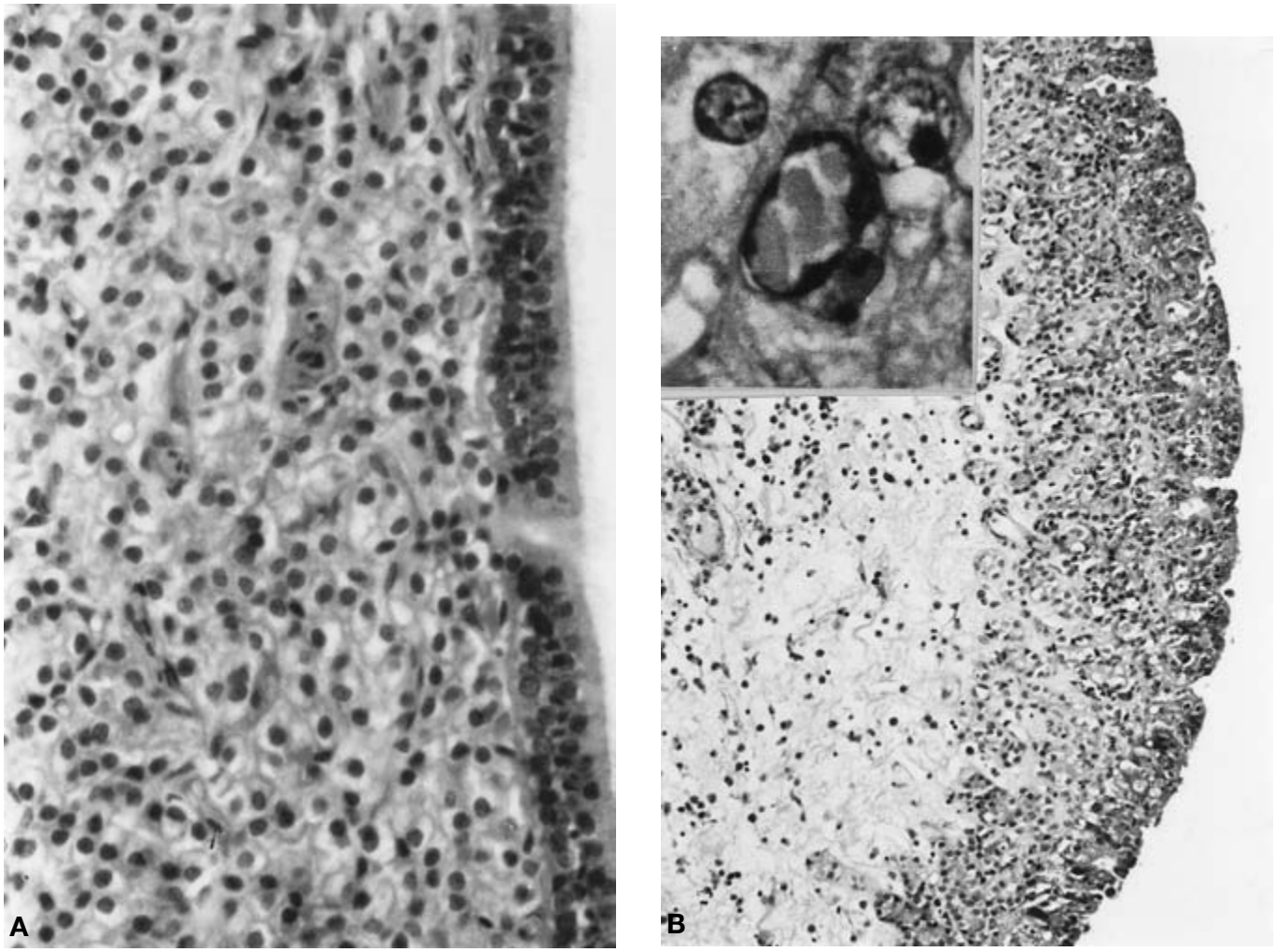
Gross Lesions

In naturally occurring outbreaks of EDS, inactive ovaries and atrophied oviducts were often the only recognizable lesions, and these were not consistently present. In one outbreak, uterine edema was observed (38). The absence of lesions reflects the difficulty in selecting birds that are actually in the acute phase of disease.

Following experimental infection with EDS virus, edema of the uterine folds, and presence of exudate in the pouch shell gland commonly occurred within 9–14 days PI (55,60). Mild splenomegaly, flaccid ovules, and eggs in various stages of formation in the abdominal cavity were also observed (60).

Microscopic Lesions

The major pathologic changes occurred in the pouch shell gland (Fig. 9.7). Virus replication occurs in the nuclei of surface epithelial cells, and intranuclear inclusion bodies were detectable from 7 days PI onward (Fig. 9.8) (55,60). Many affected cells were sloughed into the lumen, and there was a rapid and severe inflammatory response with heterophilic infiltration of the epithelium and lamina propria and mucosal edema, together with macrophages, plasma cells, and lymphocytes, in the lamina propria (Fig. 9.7, 9.8). Inclusion bodies were not seen after the third day of abnormal egg production, but viral antigen persisted for up to 1 week (55). As the lesions progressed, heterophils were less common and the mononuclear cells dominated. The sloughed surface epithelium was replaced initially by squamous to cuboidal epithelium, with rapid return to the normal pseudostratified, ciliated columnar epithelium. In some recovering and recovered birds which were producing normal eggs, a few areas



9.8. A. Normal uterine mucosa. Surface epithelium consists of a single layer of columnar cells, many of which are ciliated; underlying these are tubular glands. B. Pronounced edema of uterine submucosa, atrophy of tublar glands, and infiltration of entire mucosa by mononuclear cells are present at 8 days postinoculation (PI). Inset: Intranuclear inclusion body in superficial epithelial cell. Note margination of nuclear chromatin and three eosinophilic inclusions in the nucleus.

of cuboidal surface epithelium, and a few lymphoid aggregates or minimal loose infiltrates of lymphocytes and plasma cells persisted.

Most descriptions of the pathology of birds from naturally occurring disease outbreaks do not include the finding of inclusion bodies or the acute inflammatory and necrotizing phase of the disease. This is due to the transient nature of these lesions and the difficulty in finding affected birds among the thousands of birds which may be present in an affected flock, where not all birds will be infected simultaneously.

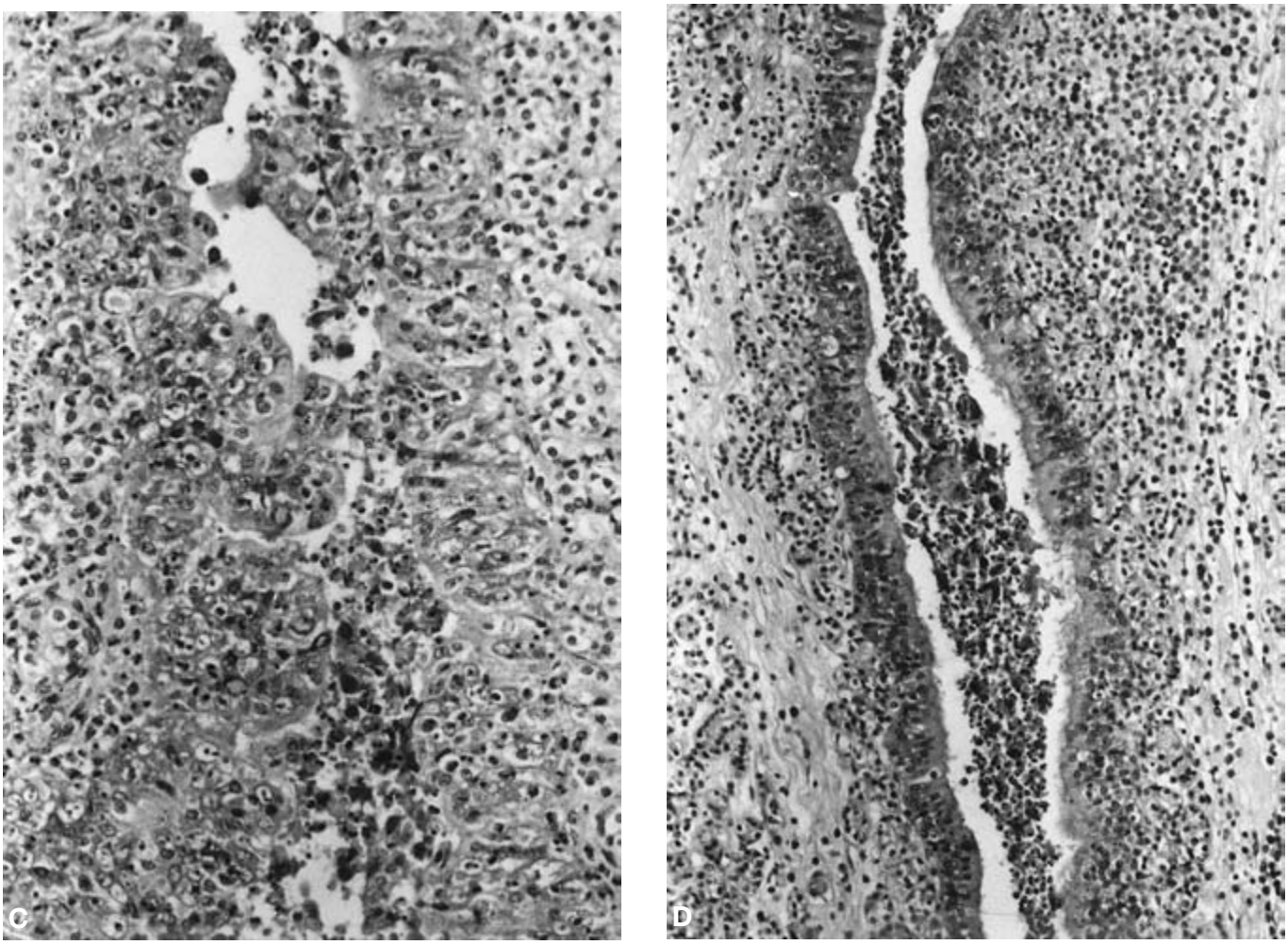
Pathogenesis of the Infectious Process

Experimental oral infection of adult hens with EDS virus resulted in a viremia with limited virus replication in the nasal mucosa (57). At 3–4 days postinfection (PI), virus replication occurred in lymphoid tissue throughout the body; particularly in spleen and thymus. In addition, the infundibulum of the oviduct was consistently affected. At 7–20 days PI, substantial levels of virus replication were detected in the pouch shell gland (Fig. 9.8) with lower levels of replication in other parts of the oviduct. This replication was associated with a pronounced inflammatory response in the pouch shell gland, and production of eggs with abnormal shells (57, 60, 70).

Unlike the aviadenoviruses and siadenoviruses of birds, EDS virus does not appear to replicate in the intestinal mucosa, and presence of virus in the feces is probably due to contamination with oviduct exudate (57).

Immunity

Following experimental infection with EDS virus, antibody was detected by indirect fluorescent antibody test (IFA), enzyme-linked immunosorbent assay (ELISA), and SN and HI tests 5



9.8. (*continued*) C. Uterine surface epithelium is markedly hyperplastic 11 days PI; there is complete loss of cilia. (Smyth) D. Exudate in uterine lumen consists of degenerating epithelial cells and heterophils mixed with mucus. Epithelium is devoid of cilia; tubular glands are almost absent; and uterine wall is infiltrated with lymphocytes and heterophils. (Smyth)

days after infection and by double immunodiffusion (DID) test after 7 days (4). Antibodies reached a peak after 4–5 weeks, and the immunoprecipitating antibodies were more transient.

Birds still excreted EDS virus in the presence of high levels of HI antibody, but some birds that excreted the virus failed to develop antibody (19).

Antibody was transferred to the embryos through the yolk sac, and young chicks had high EDS HI titers (geometric mean titers, 6–9 $\log_2$). This antibody had a half-life of 3 days (21). Active antibody production was not stimulated until the chicks reached 4–5 weeks of age, when maternal antibodies were almost undetectable (21). When the disease was being eradicated, it was found that some flocks, which were apparently free from detectable antibody, as indicated by HI testing on two or three occasions, nevertheless suddenly developed EDS. This suggested that some chicks had become infected *in ovo* and had developed a latent infection but had failed to develop antibody. With the onset of egg production, the virus then was reactivated and virus excretion took place. It is not known if all chicks developed antibody at this point, but it is possible they did not, because less than 100% of birds in infected flocks had antibody.

If a flock as a whole develops antibody to EDS virus before coming into lay, egg production will not be affected (10).

Diagnosis

Isolation and Identification of EDS Virus

The most sensitive medium for virus isolation is either embryonated duck or goose eggs derived from a flock free of EDS virus infection or duck or goose cell cultures. If these are unavailable, chicken cells should be used. Chicken embryo liver cells were more sensitive than chicken kidney cells, and chicken embryo fibroblasts were insensitive (3). Embryonated chicken eggs are not suitable. Not only are duck or goose cells or embryonated duck or goose eggs more sensitive, they also have the advantage that they do not support the growth of many chicken viruses.

It is not sufficient to rely on embryo death or cytopathic effect to indicate isolation of EDS virus. Allantoic fluid from inocu-

lated goose or duck eggs or supernatant from infected cell cultures should be checked after each passage for presence of EDS virus HA, using avian erythrocytes. (0.8% chick erythrocyte suspension is suitable). Alternatively, immunofluorescence, using a labeled EDS virus antiserum, can be used to detect the presence of the virus in the cells. Antiserum conjugated to aviadenoviruses will not detect EDS virus. If duck cells are used for isolation, a minimum of 2 passes are required, and with chick cells, 2–5 passes are necessary before declaring a specimen negative. The need for extensive passage is partly due to poor growth of the virus on primary isolation in chick cells, and partly because virus titers in the tissues can vary particularly if the bird submitted to the laboratory is not at the stage of the disease process where virus titers are maximal. The successful use of antigen capture ELISA and PCR-based tests have recently been described (20,49,52,53,67).

Selection of Specimens

Because of the absence of obvious clinical signs and the often slow spread of infection, it can be very difficult to select infected birds for either virus isolation or serology. The finding that abnormal eggs contained virus and that these eggs were produced after the bird had developed EDS antibodies has allowed a rational approach to diagnosis (56). To isolate the virus, abnormal eggs may be fed to antibody-negative adult laying hens. At the first appearance of abnormal eggs, the hens may be euthanised and virus isolation attempted from the pouch shell gland. For serological diagnosis, all the birds in cages where abnormal eggs are being produced should be blood sampled. If the birds are housed on litter, care must be taken to select samples throughout the house, as it is usually not possible to determine which birds are producing abnormal eggs in these circumstances.

Serology

The HI, ELISA, SN, DID and IFA tests are of similar sensitivity (4). When birds have been infected with a number of adenovirus serotypes however, with consequent stimulation of high levels of cross-reactive antibodies, there were positive reactions in the ELISA, IFA, or DID tests but not in the HI or SN tests (4). HI is the test of choice for serological diagnosis. Antigen for HI test can be prepared in either embryonated duck eggs or in cell culture. Higher HA titers are obtained if duck eggs are used, but high HA titers can also be obtained using chick embryo liver cell cultures. A suitable HI test uses 4 HA units of antigen, an initial 1:4 serum dilution, and 0.8% chicken erythrocytes. The EDS virus agglutinates erythrocytes from chickens, geese, turkeys, and ducks but not mammalian erythrocytes. There is no hemolysin. If nonspecific hemagglutinins are present in the serum, they can be removed by pre-adsorption of the serum with a 10% erythrocyte suspension. The SN test, using 100 $TCID_{50}$ of EDS virus, 1 hour reaction time at 37°C, and duck or chick cell cultures as the indicator system, is sensitive and specific. When using chick cell cultures, it often helps if the end-points are read by presence of hemagglutinins in the supernatant fluid rather than by cytopathology. The SN test is really required only in diagnostic situations to confirm an unusual HI test result as in an eradication program or to confirm a positive HI result in species in which EDS has not previously been recognized.

Many flocks containing birds that had been infected with EDS virus *in ovo* did not develop antibodies during the growing period; it only became apparent immediately following the development of clinical signs of the disease. Therefore, even a negative serological test of all birds in a flock, at around 20 weeks of age, gives no guarantee of freedom from infection.

Differential Diagnosis

Egg drop syndrome should be suspected whenever there is failure to achieve predicted egg production levels or if there are falls in egg production, especially if birds are healthy and eggshell changes precede or are concurrent with the decline. Shell-less eggs are usually a feature of EDS but are often missed because they may be consumed by the birds, be trampled into the litter, or fall through the wire mesh of cage floors. Therefore, an inspection should take place early in the morning before the eggs can be eaten. If the birds are housed on litter, a careful search will reveal egg membranes. Although shell-less, soft-shelled, and thin-shelled eggs are characteristic, misshapen and ridged eggs are not a feature. In an infected flock in which vertical transmission of EDS virus has occurred, most if not all cases occur around peak egg production, but any age of flock can be infected by lateral spread.

Although signs of EDS are quite characteristic, diagnosis must not be made on the clinical picture alone but should be confirmed by EDS HI test before vaccination is considered.

Intervention Strategies

Management Procedures

Because classical EDS is spread primarily by vertical transmission through the egg, replacement birds should be derived from uninfected flocks. Endemic EDS has often been associated with a common egg-packing station in which contaminated egg trays have been a major factor in the spread of the disease. Virus is also present in droppings, and lateral spread is possible because the virus is resistant to inactivation. Circumstantial evidence exists for spread by personnel and transport, and, therefore, sensible hygienic precautions are required.

The infected birds develop a viremia, and thus it is important that bleeding needles, needles for inoculating vaccines, and other equipment should be sterilized between birds.

If infected and uninfected breeding flocks exist within the same organization, separate hatcheries, staff, and transport should be used. If this is not possible, separate setters and hatchers should be used, and hatches should be scheduled to take place on different days of the week. The minimum possible precautions (which are not recommended) would be to use separate hatchers and to sex, vaccinate, and dispatch the clean stock before handling potentially infected chicks. It is particularly important to keep basic or grandparent breeding stock from an infected breed separate from noninfected birds of another breed, and these eggs should never be incubated in the same hatchery.

In certain areas of the world, particularly where the drinking

water for the birds is derived from dams, lakes, or rivers, EDS virus infection has been common. These outbreaks have been controlled either by using water from wells or by chlorination of the water. In units where ducks or geese are kept, they should be carefully segregated from the chickens. If possible, all housing should be made wild bird-proof. It is now well documented that wild ducks and wild geese are often infected with EDS virus, but it is not known how widespread infection is in other avian species.

Eradication

Egg drop syndrome was eradicated successfully from a breeding organization in Northern Ireland. The method was based on a number of presumptions: (i) chickens produced from EDS virus-infected eggs may be latently infected and fail to develop antibody; (ii) the virus will become reactivated and will be excreted at around the time of peak egg production, and EDS antibody will develop, which will prevent or reduce further excretion; (iii) lateral spread is poor.

The eradication program was based on the elite and grandparent flocks aged 40 weeks or more. At this stage, these flocks had produced abnormal eggs and had EDS HI antibody. Chicks hatched from these eggs were divided into small groups of about 100 (separated by netting wire). Ten to twenty-five percent of the chicks were HI tested for EDS antibodies by HI test at about 6-week intervals. If one or two reactors were found, they were removed; 100% of the birds in the pen and 100% of adjoining pens were then tested twice at weekly intervals. If HI test positive reactors were found or if positive reactors kept appearing within a single pen, the whole pen was then removed and the in-contact pens of birds were tested. At 40 weeks, 100% of the birds were tested by HI test, and eggs were collected for the next generation. This program was successful, and subsequently, the grandparent and parent flocks were found to be free of infection.

Vaccination

Types of Vaccine

An oil-adjuvant inactivated vaccine is widely used and gives good protection against clinical EDS. The birds are vaccinated between 14 and 16 weeks of age. If uninfected birds are vaccinated, EDS HI titers of 8–9 $\log_2$ can be expected. If the flock has been exposed previously to EDS virus, HI titers of 12–14 $\log_2$ may be found. An HI antibody response can be detected by the seventh day after vaccination, with peak titers achieved between the second and fifth weeks. Vaccinal immunity lasts at least 1 year (10,19,34,58). Although properly vaccinated birds are protected against disease and do not appear to excrete EDS virus, improperly vaccinated birds with low EDS HI titers excreted the virus following challenge (17).

Field Vaccination

When vertical or lateral transmission of EDS virus is a possibility, flocks in danger can be protected by vaccination in the growing period. If one or more houses on a multiage laying site become infected due to lateral spread of the virus, careful evaluation must be undertaken before vaccinating the healthy birds during lay. Undoubtedly, the healthy birds can be protected by vaccination, but the cost of vaccination, combined with costs and effects of the handling of the birds in order to administer the inactivated vaccine, must be carefully weighed against the economic returns achieved from the protection. It is possible to limit the spread of virus on a site by good hygiene. It is particularly important to remember that the infected egg is potentially the most dangerous source of virus.

Treatment

Various treatments have been tried (for example, vitamins and increasing calcium or protein in the ration), but in controlled trials, no effect could be demonstrated. Therefore, no successful treatment is available.

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Hemorrhagic Enteritis and Related Infections

F. W. Pierson and S. D. Fitzgerald [1]

Introduction

Definitions and Synonyms

Hemorrhagic enteritis (HE) is an acute viral disease of turkeys 4 weeks of age and older characterized by depression, bloody droppings, and death. Clinical disease usually persists in affected flocks for 7–10 days. Due however to the immunosuppressive nature of the agent, secondary bacterial infections may extend the course of illness and mortality for an additional 2–3 weeks.

Marble spleen disease (MSD) is a condition affecting confinement-reared pheasants 3–8 months of age. The causative virus is serologically indistinguishable from that of HEV and varies only slightly at the genomic level. However the clinical disease is predominantly respiratory in nature with death occurring due to lung edema, congestion, and asphyxia.

Evidence of similar infections in other gallinaceous fowl exists. In particular, a condition referred to as avian adenovirus splenomegaly (AAS) has been described in broiler breeder chickens characterized by splenomegaly, pulmonary edema, and congestion.

Economic Significance

Financial losses due to HE within the United States are purported to have exceeded $3 million per year prior to development of a vaccine (20). In 1984, losses due to HE associated *Escherichia coli* infections were estimated to be $40 million per year (108). Today, due to the extensive use of vaccines, highly pathogenic outbreaks of HE are rare in the U.S. However lapses in protection may result in primary HE or more commonly a secondary bacterial infection like colibacillosis, which is considered to be a disease of economic importance in the commercial turkey industry.

1. The authors are greatly indebted to C. H. Domermuth, W. B. Gross, and B. S. Pomeroy for their contributions to earlier editions of this subchapter.

The economic impacts of MSD in pheasants or AAS in chickens have not been assessed.

Public Health Significance

The etiologic agents responsible for HE, MSD, and AAS are not known to cause illness in humans nor has evidence of seroconversion been documented.

History

HE was first observed in Minnesota by Pomeroy and Fenstermacher (99) and later in Ohio by Gale and Wyne (44). The disease commonly known as "bankruptcy gut," reached epidemic proportions in Texas in the early 1960s and Virginia in the mid-1960s (48). It occurred in both confinement and range turkey operations and exhibited a strong tendency to infect successive flocks on the same premises. In 1966, the first reported outbreak of MSD in ring-necked pheasants was described by Mandelli *et al*. (69). AAS was identified in broiler breeder chickens by Domermuth *et al*. in 1979 (27).

Experimental transmission of HE using filtered and unfiltered intestinal contents from turkeys dying of the disease was first described in 1967 (48) and by the mid-1970s, the causative agent definitively identified as an adenovirus (8, 19, 25, 43, 47, 59, 117). Parallel studies with MSD have yielded similar results (55, 56, 58, 59).

Etiology

Classification

Morphologic, histologic, immunologic, and chloroform-resistance studies indicate that HE virus (HEV), MSD virus (MSDV), and AAS virus (AASV) are members of the family *Adenoviridae* (8, 19, 25, 43, 47, 56, 60, 118).

HEV, MSDV, and AAS were originally assigned to the genus *Aviadenovirus* and designated as avian adenovirus group (type) II (28) to distinguish them from the other members of the genus (fowl adenoviruses, group I; EDS 76, group III). This classification was based on the observation that convalescent HE antiserum from turkeys protected pheasants against MSD (25) and that MSDV and AASV were indistinguishable from HEV in agar-gel immunodiffusion tests (19, 25, 27, 28, 29, 58). As a group, they were shown to be serologically distinct from CELO virus and other turkey adenovirus isolates (28, 62). Recent DNA sequence homology data have shown HEV and MSDV to be different enough from other members of the genus to warrant reclassification (4, 14, 63, 98,). They therefore have been placed in a new genus, *Siadenovirus,* and been given one species name, turkey adenovirus A (5, 13, 14). The other member of the genus is frog adenovirus 1. The genus name is derived from an open reading frame in the early transcription region (E1) that has a high sequence homology with bacterial genes coding for sialidase. There are also several other putative genus-specific genes that share no sequence similarity with other adenoviruses (13). The functional importance of the sialidase homolog and other genus-specific genes has yet to be determined. It has been speculated that the siadenoviruses originated in amphibians and then adapted to avian species (15).

Morphology

Ultrastructure

Thin-section tissue preparations examined by electron microscopy reveal that HE and MSD viral particles are non-enveloped icosohedrons, with a total capsomere count of 252. They occur in empty and dense forms and are arranged intranuclearly in loosely packed aggregates or crystalline arrays (8, 43, 56, 60, 118, 127). Only one penton fiber appears to be present at each vertex (124) which distinguishes them from members of the genus *Aviadenovirus* that possess two fibers (73).

Size, Density

In early studies, HEV was found to readily pass through filters with porosities of 220 and 100 nm but not 10 nm (17, 48). Electron microscopy of intranuclear inclusion bodies and cesium chloride-purified preparations reveal virions to be 60–90 nm in diameter (9, 56, 59, 60, 118), but differences in size are probably within the limits of experimental error. HEV and MSDV are reported to have buoyant densities between 1.32–1.34 g/mL (8, 55, 57, 124, 127)

Chemical Composition

HEV, MSDV, and AASV are linear, double stranded DNA viruses (14, 55). Complete sequences (Genbank Accession Numbers AF074946, AY849321) and maps of the HEV genome have been published for both virulent and avirulent strains (4, 13, 98,). The genome length is approximately 26.6 kb, which ranks it as one of the shorter adenovirus genomes (15). Guanine and cytosine comprise 34.9% of the bases, which is low by comparison with other adenoviruses (98, 4). However, sixteen genes appear to be conserved based on comparative analysis with a variety of DNA sequences from other members of the family *Adenoviridae* (15).

An extensive list of putative HEV proteins has been published (14). Polyacrylamide gel electrophoresis and Western blotting techniques suggest that HEV and MSDV have at least 11 distinct structural polypeptides with molecular weights ranging between 14 and 97 kD (82) and 9.5 and 96 kD (124). Six of these proteins have been further characterized. They include a 96 kD polypeptide believed to be a monomer of the major outer capsid or hexon protein, 51/52 kD and 29 kD polypeptides believed to be the vertex penton base and fiber proteins, a 57 kD homologue of human adenovirus group 2 IIIa protein, and two core nucleoproteins of 12.5 kD and 9.5 kD each (124).

Virus Replication

Early electron micrographic studies suggested that HEV and MSDV replication took place in nuclei of reticuloendothelial cells (9, 43, 60, 118, 127). Adherent mononuclear macrophages and nonadherent mononuclear cells bearing IgM have since been reported to support viral replication (111, 112, 123). Thus, macrophages and B lymphocytes are considered to be the primary target cells (111, 112).

Enzyme-linked immunosorbent assay (ELISA), immunofluo-

rescent and immunoperoxidase staining and polymerase chain reaction (PCR) have revealed the presence of infected cells in a variety of tissues including intestine, bursa of Fabricius, cecal tonsils, thymus, liver, kidney, peripheral blood leukocytes, lung, and spleen (4, 35, 40, 52, 94, 108, 112, 119). However, on the basis of immunodiffusion and immunoperoxidase studies, the spleen appears to be the major site of viral replication (20, 107).

The replication strategy at the cellular level is presumed to be similar to that of other adenoviruses. Infection begins by viral attachment facilitated by the fiber and penton base proteins. Receptor-mediated endocytosis then occurs. DNA transcription takes place in the nucleus and utilizes host cellular RNA polymerase II. Genome replication also occurs in the nucleus and involves virus-encoded DNA-dependent DNA polymerase and the formation of a pan-handle intermediate with base pairing occurring at inverted terminal repeats. Virions are assembled in the nucleus and released upon cell disintegration (14).

Susceptibility to Chemical and Physical Agents

Infectivity of HEV can be destroyed by heating at 70°C for 1 hour; drying at 37° or 25°C for 1 week (17); or by treatment with 0.0086% sodium hypochlorite (18). Sodium lauryl sulfate, phenolic and iodine based disinfectants are also known to be effective (17). Treatment with 50% chloroform or 50% ethyl ether does not alter infectivity (20) nor does heating at 65°C for 1 hour, wet storage for 4 weeks at 37°C, 6 months at 4°C, or frozen storage for 4 years at –20°C. The virus is also stable at low pH (20).

Strain Classification

Traditionally, HEV, MSDV, and AASV strains have been classified according to their host source i.e., turkeys, pheasants, or chickens. Antigenic differences have been reported based on monoclonal antibody affinity (122, 129) but strains are considered to be serologically indistinct and provide cross protection (22, 25, 26, 27, 30, 58). A comparison of the genomes for virulent and avirulent strains of HEV indicates that they are 99.9% identical (4). However, the occurrence of mutations in the penton fiber, open reading frame 1 (ORF1), and/or E3 genes may account for variations in virulence (4). Strain differences based on restriction endonuclease fingerprinting have been reported (128) but the method appears to be unreliable which is not surprising given the high level of sequence homology. Finally, it is not uncommon for HEV isolates to be referred to as virulent or avirulent based on the severity of lesions they produce in turkeys i.e., splenomegaly, gastrointestinal hemorrhage, and death in the case of the former and splenomegaly alone in the case of the latter.

Laboratory Host Systems

For laboratory purposes, HEV is commonly propagated in 6-week-old turkey poults via intravenous (IV) or oral inoculation with splenic tissue derived from infected birds diluted 1:1 v/v with phosphate buffered saline (PBS). Spleens from these birds can then be harvested 3 or 5 days postinoculation (DPI) respectively and frozen. Inoculation of specific-pathogen-free turkey eggs with MSDV on day 24 of embryonation also results in infection and replication with peak viral loads occurring in the spleen, intestine, and liver 6 DPI (1). Although theoretically possible, attempts at *in vitro* propagation of HEV in isolated spleen cells have been unsuccessful. Perrin *et al.* (89) inoculated turkey splenocytes with HEV and were able to recover virus but unable to confirm viral replication. Likewise, Fasina and Fabricant (35) demonstrated *in vitro* infection of chicken, turkey, and pheasant splenocytes by immunofluorescence but could not demonstrate viral release. It was not until 1982 that successful *in vitro* propagation was achieved by Nazerian and Fadly (79) who demonstrated serial passage of HEV and MSDV in a turkey lymphoblastoid B cell line derived from a Marek's disease virus associated tumor. The cell line, known as MDTC-RP19, has since become the standard system for *in vitro* HE vaccine production. An *in vitro* method using purified peripheral blood leukocytes from turkeys has also been described (123).

Pathogenicity

Mortality in field outbreaks of HE is reported to vary from more than 60% (48) to less than 0.1%. Experimentally where spleen size and the presence of precipitating antigen indicated 100% infection, mortality was found to range between 80% for the most pathogenic strain and 0% for the least. Present information suggests that the ability of a given strain to produce mortality is a fairly stable characteristic. Only one instance of possible reversion to virulence of an avirulent strain has been reported (96).

Mortality rates in pheasants naturally infected with MSDV have been reported to be 5–20% over a period of 10 days to several weeks (72). As with HEV, variations in pathogenicity among MSDV isolates would be expected. However pheasants experimentally infected with cell-culture propagated MSDV or virulent and avirulent HEV showed typical gross and microscopic splenic lesions but no lung lesions or mortality (30). It has been suggested that other environmental factors may be involved in occurrence of lung lesions and mortality in field outbreaks of MSD (30).

Pathobiology and Epidemiology

Incidence and Distribution

Hemorrhagic enteritis has been a serious problem in at least 10 states in the U.S. and observed throughout the world wherever turkeys are raised (20). Serologic surveillance data suggest that infection with HEV is widespread among adult turkeys (92). MSDV has been documented in confinement pheasant operations throughout the U.S., Canada, Europe, Australia, and Korea (6, 68, 69, 72, 101, 110, 113, 114, 115). Similarly, a high incidence of antibody in mature chickens suggests a wide distribution of AASV (29).

Natural and Experimental Hosts

Until recently, turkeys, pheasants, and chickens were the only known natural hosts for HEV and related viruses. It is now thought that guinea fowl (11, 71) and psittacines (45) may also be naturally infected. With regard to wild birds, a serologic survey of 42 different species revealed no evidence of infection outside the order Galliformes (16). Even wild populations of turkeys ap-

pear to be at little risk (51) due to their elusive nature. When infection does occur, host genetics appear to influence the severity of clinical disease and lesion formation in both turkeys (67) and pheasants (64). Laboratory experiments indicate that HEV isolates from turkeys will infect ring-necked pheasants and MSDV isolates from ring-necked pheasants will infect turkeys (26). Chicken isolates will also infect turkeys (27, 28, 29). Lesions have been produced in a variety of other gallinaceous species including golden pheasants, peafowl, chickens, and chukars by experimental infection with HEV (20). However death has not been reported in species other than the natural hosts.

Age of Host Most Commonly Affected

Primarily due to the protection afforded by maternal antibody (32), HE is not typically seen in turkeys until about 6 weeks of age, with most field cases occurring between 6–11 weeks of age (44, 100). A single case in 2.5-week-old poults has been reported and was thought to have been associated with a lack of maternal antibody (49). Newly hatched, seronegative poults have been shown to be susceptible to infection but refractory to intestinal lesion formation (79, 31) which suggests that some sort of target cell maturation may be necessary for the development of fulminant disease (32).

Marble spleen disease in pheasants occurs naturally in birds 3–8 months of age (6, 72). Those younger than 4 weeks of age appear to be less susceptible to infection either due to the presence of undetectable, yet effective levels of maternal antibody or an insufficiency of target cells (39).

In chickens, field infection with AASV has been observed in broiler breeders 20–45 weeks of age (27, 29).

Transmission, Carriers, and Vectors

HEV can be transmitted by oral or cloacal inoculation of susceptible poults with infectious feces (48, 61). Virus can remain viable for several weeks in carcasses protected from drying or in wet fecal material. HEV has also been recovered from contaminated litter and the disease is known to reoccur in houses where it has appeared before (20). Recent data suggest the development of persistent infection in recovered birds (4). However there is no epidemiologic evidence of egg transmission or true biological vectors (20). Therefore it is likely that HEV, MSDV, and AASV are transmitted mechanically from actively or persistently infected birds to susceptible ones.

Incubation Period

In turkeys clinical signs and mortality begin about 5–6 days after oral or cloacal and 3–4 days after IV inoculation with splenic extracts containing HEV (24). The incubation periods following oral inoculation with MSDV in pheasants (25) and AASV in chickens (27, 126) appear to be 6 and 5–7 days respectively.

Clinical Signs

HE is characterized by a rapid progression of clinical signs over a 24 hr period (20, 99). These include depression, bloody droppings, and death. Feces containing frank blood are frequently present on the skin and feathers surrounding the vents of moribund and dead birds. Bloody feces may also be forced from the vent if moderate pressure is applied to the abdomen. In naturally infected flocks, signs of disease tend to subside within 6–10 days of the appearance of bloody droppings. In pheasants infected with MSDV, death is often acute. Signs if present consist of depression, weakness, and progressive dyspnea. Occasionally, a pre-mortem nasal discharge is noted (37). The presentation for AASV in chickens is similar to that of pheasants but generally less severe (27, 29).

Morbidity and Mortality

In field outbreaks of HE, all or nearly all birds appear to be infected, as indicated by seroconversion (24) and resistance to subsequent experimental challenge. Depressed, clinically affected poults usually die within 24 hours or recover completely. Field mortality ranges from less than 1 to slightly more than 60% with the average being 10–15%. Mortality of 80% is often seen in laboratory experiments where 100% infection is achieved. Morbidity associated with MSD and AAS is likely to be similar to that of HE. Mortality in MSDV-infected pen-reared ring-necked pheasants has been reported to be 2–3%, but may reach as high as 5–20% over a course of 10 days to several weeks (9, 20, 62, 72). In mature chickens with AAS, mortality as high as 8.9% has been reported (29).

Pathology

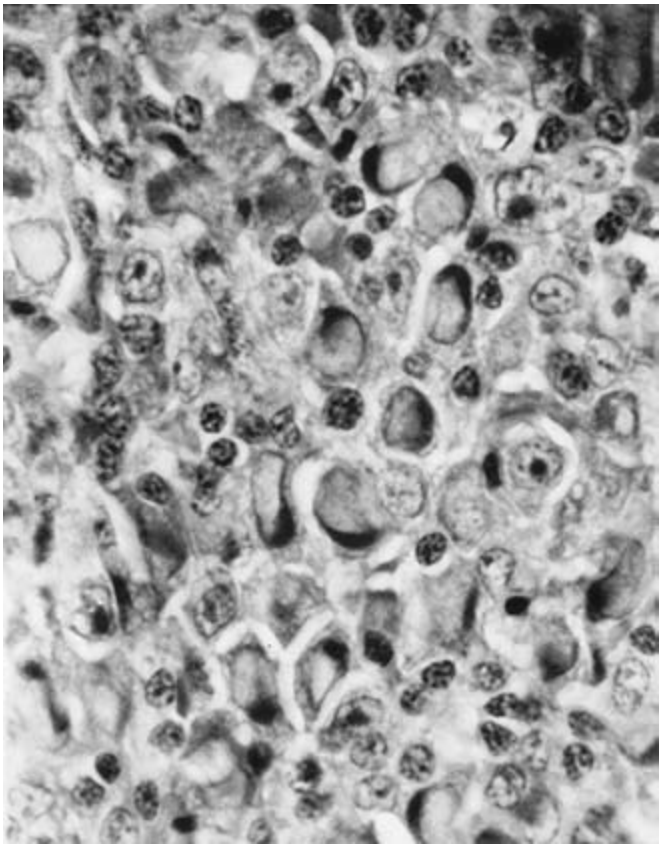
Gross

Dead poults routinely appear pale due to blood loss but are often in good flesh and have feed in their crops. The small intestine is commonly distended, grossly discolored, and filled with bloody contents (Fig. 9.11F). The intestinal mucosa is congested and in some individuals, covered with a yellow fibrinonecrotic membrane. Lesions are usually more pronounced in the proximal small intestine (duodenal loop) but can extend distally in severe cases. Spleens of infected birds are characteristically enlarged, friable, and mottled in appearance (Fig. 9.11G); however, those of dead poults tend to be smaller presumably due to blood loss and subsequent contraction. Lungs may be congested, but other organs are generally pale. Enlarged livers and petechial hemorrhages in various tissues of dead poults have also been reported post mortem, but these findings are too inconsistent to be of diagnostic value (8, 43, 44, 46, 60, 100). Lesion formation with virulent strains appears to be dose dependent (80, 88).

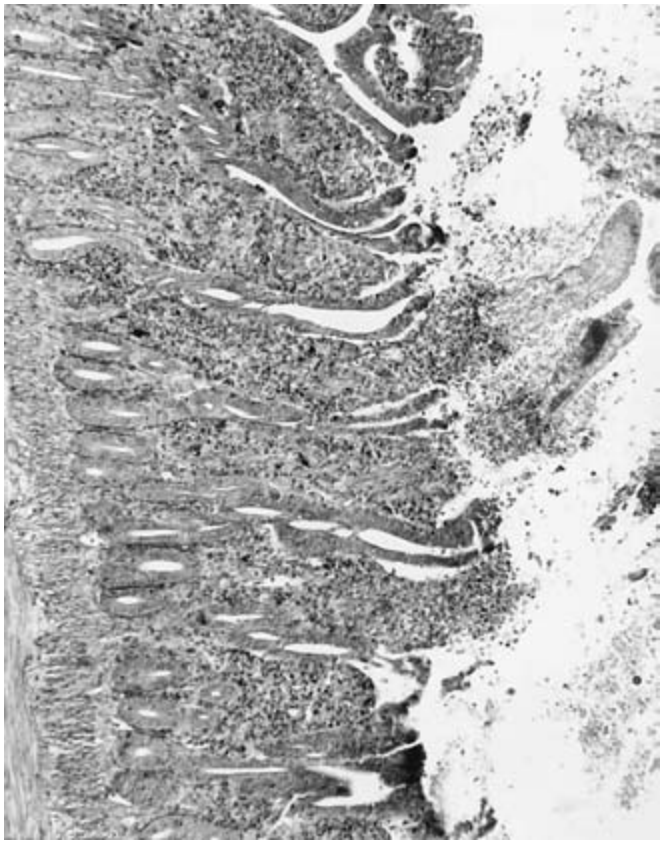
Gross lesions in pheasants infected with MSDV consist of enlarged, mottled (marbled) spleens and edematous congested lungs (6, 72). Intestinal lesions have not been noted. In broiler breeder chickens infected with AASV, gross splenic and lung lesions resemble those of MSDV in pheasants (27, 29).

Microscopic

Pathologic changes that characterize HE are most evident in the immune and gastrointestinal systems. Splenic lesions present at death (Fig. 9.9) include hyperplasia of white pulp and lymphoid necrosis. Intranuclear inclusions can be found within mononuclear cells i.e., macrophages and lymphocytes (75, 107, 112). Proliferation of white pulp surrounding splenic ellipsoids is evident as early as 3 DPI. This leads to large, irregular, confluent is-



9.9. Section of spleen of turkey affected with hemorrhagic enteritis. Nuclei of infected cells contain characteristic intranuclear inclusions. H & E, × 550



9.10. Section of small intestine of turkey affected with hemorrhagic enteritis. Lesions include severe congestion of the mucosa, degeneration of epithelial cells covering tips of villi, sloughing of epithelial cells and tip of villus, and hemorrhage into the lumen. H & E, × 550.

lands of white pulp which are grossly visible as mottling 4–5 DPI (107). Hematoxylin and eosin (H&E) and immunoperoxidase staining reveal numerous intranuclear inclusions in these zones between 3 and 5 DPI. By 4–5 days PI white pulp begins to undergo necrosis and by 6–7 DPI it has completely involuted with only occasional plasma cells appearing in the red pulp (107). In addition to splenic changes, lymphoid depletion is also noted in both the cortical and medullary areas of the thymus (56) and bursa of Fabricius (56, 107) between 3 and 9 DPI.

Typical lesions in the gastrointestinal tract include severe congestion of intestinal mucosa, degeneration and sloughing of villus epithelium, and hemorrhage in the villus tips (Fig. 9.10). Hemorrhage is believed to result from endothelial disruption rather than destruction because blood vessels in the lamina propria appear intact and red cells are observed moving out of these vessels by diapedesis (107). Increased numbers of mononuclear cells positive for intranuclear antigen are observed in the lamina propria (52) in addition to mast cells (86), plasma cells, and heterophils (107). These histopathologic changes are most pronounced in the duodenum just posterior to the pancreatic ducts, but similar less severe lesions may also occur in the proventriculus, gizzard, distal small intestine, ceca, cecal tonsils, and bursa of Fabricius (20). Additionally cells containing intranuclear inclusions can be seen in the liver, bone marrow, peripheral blood leukocytes, lung, pancreas, brain, and renal tubular epithelium (8, 43, 46, 52, 60, 75, 119).

MSDV and AASV produce intranuclear inclusions and splenic lesions similar to those of HEV but without significant gastrointestinal involvement. In naturally occurring cases of MSDV and AASV flooding of the atria and tertiary bronchi with fibrin and red cells as well as vascular congestion are observed in the lung (6, 29, 40). However, these lesions are not commonly seen with experimental infections. Typical intranuclear inclusions can be found in the liver, lung, kidney, bursa, and bone marrow. However, no inclusions are noted in the gastrointestinal tract (37, 40). Immunohistochemical staining for MSDV antigen on sections of lung from naturally infected pheasants reveals moderate numbers of positively stained nuclei in mononuclear cells within atria (40). Whether these are infected peripheral blood leukocytes similar to those seen with HEV or a finding of significance in the pathogenesis of the pulmonary edema with MSD is unknown.

Pathogenesis of the Infectious Process

HEV and related viruses are considered to be lymphotropic and lymphocytopathic (38, 52, 107, 123) with IgM bearing B lymphocytes being the primary target (105, 112). Macrophages can also

appear to support viral replication (111, 123). Given this it is not surprising that bursectomy impairs viral replication and lesion formation (31, 38, 111) or that marked depletion of IgM bearing cells in the spleen and peripheral blood occurs during the acute phase of HEV infection (105, 111). HEV and MSDV are also known to produce transient inhibition of antibody responses to sheep erythrocytes (36, 76) and Newcastle disease virus (77) as well as suppression of B and T cell mitogenic responses *in vitro* (36, 76, 77, 78).

Multiple hypotheses regarding the immunopathogenesis of HE and related viruses have been proposed (31, 52, 74, 86, 87, 92, 103, 106, 107, 111, 112). Based on the work of numerous authors, Rautenschlein and Sharma (104) have suggested the following composite model. After oral exposure, HEV either undergoes an initial round of replication in B lymphocytes located in the intestine and bursa of Fabricius, or it travels directly to the spleen via the peripheral blood. There it infects more B cells and macrophages and replicates to high numbers. This results in an influx of CD4+ T cells and macrophages into the white pulp, presumably in an attempt to clear virus, and accounts for the hyperplasia observed during the acute phase of infection. Once activated, macrophages produce a variety of cytokines including interleukin (IL)-6 and tumor necrosis factor (TNF). These induce T cells to produce interferons (IFN) and TNF. Type II IFN activates the macrophage population, stimulating production of nitric oxide, which has antiviral, immunosuppressive properties. It also appears that type I IFN may be produced in an attempt to limit viral replication (106). As the infection process continues, HEV replication induces apoptosis and necrosis of target cells, thus depleting the IgM bearing B cell population. Cytokines released by activated T cells and macrophages also potentially induce apoptosis of by-stander cells. The result is a massive apoptotic event accompanied by a transient period of immunosuppression. It also has been proposed that the release of large amounts of cytokines initiates systemic shock and fosters the development of characteristic vascular lesions in the gastrointestinal tract, the proposed primary shock-responsive organ in the turkey.

As it applies to intestinal lesion formation, this mechanism of HEV pathogenesis is corroborated by additional evidence that suggests that histamine and prostaglandin play a role in lesion formation (85, 86). The presence of few infected cells in the intestine (112) and the ability of cyclosporin A, a T cell inhibitor, to abrogate intestinal hemorrhage also suggest that gastrointestinal lesion formation is immune mediated (111). If this model holds true, it is likely that the pulmonary lesions seen with MSDV and AASV in pheasants and chickens may be related to species differences in primary shock-responsive organs.

Presumably, as a result of the described mechanism of immunosuppression, HEV alone (66,109) or in combination with other agents like *Bordetella avium,* Newcastle disease virus (NDV), and *Mycoplasma meleagridis* appears to predispose turkeys to secondary infection with *E. coli* in the field (91). Similar findings have been reported under laboratory conditions with avirulent strains of HEV (65, 66, 83, 95). Paramyxovirus-2 and *Chlamydiophila psittaci* infections have also been observed following HEV exposure (2).

Surprisingly, improved weight gains and reduced oocyst shedding have been found in turkeys concomitantly infected with HEV or MSDV and *Eimeria meleagrimitis* (84). Likewise, dual vaccination with NDV and HEV appears to have a peculiar effect i.e., NDV antibody production is enhanced while HEV antibody production is suppressed. The spleens in this case also exhibit a more pronounced hyperplasia of the white pulp and an increased rate of apoptosis (103).

Finally, immunosuppression appears to occur with virulent as well as avirulent strains of HEV (66, 74, 90). Therefore although virulent strains may certainly be considered pathogenic, avirulent strains should not be considered completely apathogenic.

Immunity

Active

Poults recovering from natural or experimental infections with HEV are refractory to challenge. Protection does not appear to be strain specific. Strains that cause less than 1% mortality induce sufficient immunity to prevent infection with those producing much greater mortality (22). Antibodies against HEV may be detected as early as 3 DPI by ELISA (121). Such immunity appears to be long lasting if not life-long. One flock monitored over a 4–year period demonstrated a seroconversion rate of 100% at 4 week PI and was still found to be 83% positive 40 months later (21). The occurrence of life-long immunity is believed to be the result of persistent infection since viral DNA can be detected in numerous tissues up to 70 DPI despite high levels of circulating antibody (4).

Cell-mediated immunity undoubtedly plays a role in active protection against HEV and MSDV infections and lesion formation, but its role is not fully understood. Inoculation of turkeys with HEV causes an increase in splenic CD4 bearing T cells 4–6 DPI (104, 111) and elevations of CD8 bearing cytotoxic/suppressor T cells are also reported to occur at 8–10 and 16 DPI (90, 111). Selective *in vivo* T cell depletion with cyclosporin A seems to enhance splenic lesion formation and viral replication in MSDV infected pheasants (42) but the same effect has not been observed in HEV infected turkeys (111).

Passive

Maternal antibody can provide protection from clinical HE for up to 6 weeks post-hatch and has been reported to interfere with vaccination for up to 5 weeks (32). However in a commercial setting, maternal antibody typically declines enough by 3.5–4 weeks to permit vaccination with splenic HEV vaccine. Passive immunity can also be conferred by injection of birds with convalescent antiserum obtained from recovered flocks. In laboratory experiments 0.5–1.0 mL of antiserum prevented all gross lesions and as little as 0.1–0.25 mL prevented intestinal lesions (19). Administered in this fashion, hyperimmune serum has been shown to afford protection from lesion formation for up to 5 weeks (32).

Diagnosis

Isolation and Identification of Causative Agent

Large concentrations of HEV can be found in bloody intestinal contents or splenic tissue obtained from dead or moribund poults (48). Splenic material from MSDV-infected pheasants and

AASV-infected chickens is also a suitable source of virus. For all three agents, HEV seronegative turkeys preferably 6 weeks of age, can be inoculated *per os* (PO) with intestinal contents or, PO or IV with a splenic homogenate (1:1 v/v spleen:PBS). Death often occurs about 3 days after IV and 5–6 days after PO inoculation with virulent isolates. Poults that do not die as a result of infection usually have enlarged mottled spleens with typical intranuclear inclusions. Ample virus can be obtained from the spleen to perform precipitin reactions or molecular diagnostics. Sera obtained at these times also contain virus (24). Alternatively, MDTC-RP19 cells (American Type Culture Collection, Manassas, VA) grown in 65% McCoy's 5A and Leibowitz L-15 with L-glutamine media (combined 1:1 v/v), 5% tryptose phosphate broth, 20% chicken serum (HEV negative), and 10% fetal bovine serum, with 100,000 IU of penicillin/streptomycin per L can be inoculated with filtrates (.22 μm) of splenic material to isolate and propagate the viruses(79, 80).

Positive identification of HEV, MSD, and AASV is commonly accomplished through the use of an Agar Gel Immunodiffusion (AGID) method in which splenic tissue (fresh or frozen) is diluted 1:1 v/v with PBS and precipitated against polyclonal anti-HEV serum (23, 24, 93). Viral antigen can also be identified in thin sectioned (4–6 μm) frozen or formalin fixed tissues using immunofluorescent (35) or immunoperoxidase staining methods (40, 41, 53, 107). The availability of genomic sequencing data has permitted the development of standard (4, 50, 94, 98), nested, and real time (4) PCR assays for detection of viral DNA in fresh or frozen tissues. Drying of crude splenic material or DNA extracts on filter paper has also been shown to be an adequate method of storage (98). For standard PCR, DNA extraction can be performed using commercially available kits. DNA polymerase, $MgCL_2$, deoxynucleotide triphosphates (dNTPs), oligonucleotide primers, sterile de-ionized water, and sample DNA are combined in a total reaction volume of 25–50 μL. Primers which amplify a 270 base pair region of the hexon gene have been found to be reliable (nHEVL, 5′-gtg gtt cag cag aaa gtt ctt-3′; nHEVR, 5′-cag tag act cat aag caa cta t-3′). A standard 3 step thermocycler program is run for 35 cycles (4). Less commonly used methods for antigen detection include antigen-capture ELISA (54, 81, 108, 121) and *in situ* DNA hybridization (112).

Serology

Hemorrhagic enteritis virus antibodies can be detected in plasma or serum of recovered birds 2–3 weeks PI by AGID using known positive splenic material diluted 1:1 v/v with PBS as the test antigen (23, 24, 93). It is advisable to run both acute and convalescent sera if a diagnosis is to be made based on serology. Maternal antibody may be detected using AGID, but this method generally lacks sufficient sensitivity beyond 1 week of age (121). More sensitive ELISA techniques have been developed (12, 54, 80, 81, 121). These are capable of detecting maternal antibody in turkeys up to 4–6 wk of age, although most birds in the field are seronegative by 3 weeks of age (13, 121). An active immune response can be detected as early as 3 DPI (121). HEV ELISA kits are available commercially and should be useful for MSDV and AASV as well.

Differential Diagnosis

In turkeys an enlarged mottled spleen without demonstrable HEV antigen on AGID and the absence of intestinal bleeding warrants consideration of reticuloendotheliosis or lymphoproliferative disease as differential diagnoses. Enlarged, congested spleens in turkeys are often mistakenly attributed to HEV, but are commonly the result of bacterial septicemias e.g., colibacillosis, salmonellosis, and erysipelas. Additional signs and lesions usually accompany these diseases. Gastrointestinal bleeding and mucosal hyperemia may be associated with acute septicemic, viremic, or toxemic conditions. However these would rarely be observed without other lesions or signs consistent with the etiology. Coccidiosis and toxic substances e.g., heavy metals and chemicals, should also be considered.

Pheasants and chickens that die acutely with signs of respiratory distress but without enlarged mottled spleens should be tested for other respiratory pathogens including Newcastle disease virus, avian influenza virus, infectious laryngotracheitis virus, and in the case of chickens, infectious bronchitis virus. Respiratory signs with splenic enlargement and congestion should prompt consideration of bacterial pathogens like *Pasturella* and *E. coli*. Carbon monoxide, carbon dioxide, and natural gas should also be considered in confinement operations. Splenic enlargement and mottling without evidence of MSDV or AASV antigen on AGID should warrant histopathologic evaluation for neoplastic diseases such as Marek's disease, lymphoid leukosis, or reticuloendotheliosis. In chickens, hepatitis E should also be a consideration.

Intervention Strategies

Management Procedures

Effective prevention and control of HE, MSD, and AAS begin with adherence to Best Management Practice guidelines, especially biosecurity protocols, because movement of infectious litter or feces from flock to flock is the most common mode of transmission. Contaminated facilities may be cleaned and then disinfected with 0.0086% sodium hypochlorite solution or other common viricidal agents plus drying at 25°C for 1 week (17, 18). However in most commercial operations, especially those having multiple ages of birds, total elimination of the virus is considered impractical. In such cases vaccination remains the most effective means of disease control and prevention.

Vaccination

Types of Vaccine

Avirulent isolates of HEV and MSDV have been successfully used as live, water-administered vaccines (22). Two forms of vaccine are currently in widespread use for turkeys. One is a crude homogenate prepared from spleens (1:1 v/v spleen:PBS) of 6-week-old turkeys inoculated PO or IV with HEV avirulent I (Domermuth strain) or HEV avirulent II. The other is produced *in vitro* using MDTC-RP19 cells in suspension culture (33). Both vaccines appear to produce adequate seroconversion and protection (3) and are used extensively in the United States, but only the latter is commercially available. A third method of vaccine pro-

duction involving propagation of HEV avirulent I in peripheral blood leukocytes has also been described (123) and was temporarily used in Canada. A recombinant fowl pox vaccine expressing native HEV hexon has been shown to prevent mortality and gross intestinal lesions in challenged turkeys and appeared to produce less immunosuppression when compared to virulent HEV or commercial tissue culture-attenuated HEV as measured by lymphoblastogenesis assays(7). Other vaccines, including a purified hexon subunit (125) and a recombinant penton fiber-knob subunit, have been developed and are known to confer protection (97). The use of transgenic plants (tobacco) as feed grade, oral delivery vehicles for recombinant HEV fiber has also been explored (116,117).

Field Vaccination Protocols and Regimes

Successful *in ovo* vaccination of specific-pathogen-free turkeys has been described (1), but water vaccination of healthy turkeys is currently the method of choice and is usually performed between 3.5 and 6 weeks of age. The addition of a vaccine stabilizer to the water and the elimination of any water-line disinfectants including chlorine is essential to the survival of the virus and successful vaccination. Interestingly, stress applied on the day of vaccination e.g., social disruption through the movement of birds, appears to enhance the response to vaccination, either because it stimulates cell mediated immunity or permits more efficient viral replication (74). Flocks experiencing less than 100% protection from initial vaccination usually are protected by lateral transmission within 2–3 weeks. Despite this, a second vaccination 1 week after the first is occasionally employed. Not surprisingly, turkeys which have previously been exposed to immunosuppressive diseases such as avian pneumovirus exhibit a reduced response to vaccination (10).

Live, avirulent, water-administered vaccines are also effective for controlling MSD of pheasants (26, 30) but none are commercially available in the US. Vaccines for AAS of chickens have not been developed.

Treatment

Prior to the advent of effective vaccines, HE was treated by subcutaneous or intramuscular injection of 0.5–1.0 mL of convalescent antiserum obtained from healthy flocks at slaughter (19). Treatments have not been described for MSD of pheasants or AAS of chickens. However, due to the immunosuppressive nature of HE and related viruses, treatment for secondary bacterial infections, primarily colibacillosis, must be considered. Selection of an appropriate antimicrobial based on culture and sensitivity is always advised. Correction of management deficiencies and vaccination for other primary agents that may be exacerbated by exposure to field or vaccine challenge with HEV e.g., *Bordetella avium* and Newcastle disease virus, must not be neglected.

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Quail Bronchitis

Willie M. Reed and Sherman W. Jack

Introduction

Quail bronchitis (QB) is a naturally occurring, acute, highly contagious, fatal respiratory disease of young bobwhite quail (*Colinus virginianus*). The disease is of major economic significance to gamebird breeders and has a worldwide distribution (1, 2). Quail bronchitis is characterized by rapid onset, high morbidity, and high mortality and mainly affects captive-reared birds. The etiologic agent is QB virus (QBV). Quail bronchitis and chicken embryo lethal orphan (CELO) viruses—both serotype I avian adenoviruses—are considered to be the same agent and are not distinguishable using conventional techniques (3, 20). Both viruses produce similar disease and lesions in bobwhite quail and chicken embryos.

Few type I avian adenoviruses, other than QBV and CELO, have been evaluated for pathogenicity in bobwhite quail, with the exception of Indiana C adenovirus. Recent studies (11) have demonstrated that young bobwhite quail are susceptible to infection with Indiana C adenovirus, and the clinical disease and pathologic manifestations are indistinguishable from both naturally occurring and experimental infection with QBV.

Although QBV is infectious for domestic poultry, including chickens and turkeys, as well as other avian species, resulting in seroconversion, the infection is generally asymptomatic. Although QB/CELO virus has induced neoplasms in laboratory animals, there is no known public health significance (15).

History, Incidence, and Distribution

Quail bronchitis was first described by Olson (16) from a 1949 outbreak in West Virginia. A similar disease in quail had been reported as early as 1933 by Levine, however, and an agent similar to QBV was isolated by Beaudette in 1939. Following Olson's report, several outbreaks were reported in Texas in 1956–1957 and in Virginia in 1959 (4, 5). Infection occurred in 3-week-old to mature bobwhite quail, with mortality in some pens reaching 80%. Chukar partridges on the gamebird farm did not develop the disease. Circumstantial evidence indicated transmission of QBV from inapparently infected chickens or captive gamebirds other than quail to the affected bobwhite quail.

Since the early descriptions, QB has been frequently diagnosed as the cause of mortality in captive-reared bobwhite quail. The true incidence and distribution of infection are unknown, but asymptomatic infection in older birds is believed to be widespread. Infection had not been identified in wild bobwhite quail until 1981, when King *et al.* (14) reported antibodies against serotype 1 avian adenovirus in 23% of mature, free-ranging bobwhite quail collected from a research station.

Etiology

Quail bronchitis is caused by an avian adenovirus. It contains a DNA genome and is icosahedral, nonenveloped, and ranges in size from 69–75 nm in diameter (6). Based on virus neutralization, QBV is a group I/serotype 1 adenovirus indistinguishable from the Phelps strain of CELO virus (8, 15, 20). QBV/CELO serves as the type strain for group I/serotype 1 avian adenoviruses. Other techniques have been used to classify avian adenoviruses (e.g., physicochemical properties, hemagglutination, and restriction endonuclease mapping), but they have failed to further clarify the taxonomy of these agents. As with other adenoviruses, avian adeno-associated virus (AAAV) may occur with QBV (23).

Laboratory Hosts and Pathogenicity

Quail bronchitis virus is readily propagated in embryonating chicken eggs and in cultures of chicken kidney or liver cells. Although QBV will grow in chicken fibroblasts, this system is less suitable for cultivation because virus multiplication is poor. Propagation may be interfered with by concurrent AAAV infection (15, 23) or by maternal antibodies in yolk of embryonating eggs (21, 22).

In most diagnostic laboratories, initial isolation is performed in embryonating chicken eggs, sometimes requiring several blind passages before typical lesions and mortality patterns develop. A common and proven route of inoculation is via the allantoic cavity. High yields of virus can be detected in allantoic fluid 48–96 hours postinfection (PI). Isolation and propagation of QBV using the yolk sac route in antibody-free embryonating eggs is also an effective method. Infection of the embryo by the yolk sac or allantoic cavity results in dwarfing, curling, and stunting of the embryo in 2–4 days. Examination of affected embryos reveals widespread congestion and hemorrhage and enlargement of the liver, with varying degrees of necrosis and hepatitis with intranuclear inclusion bodies.

Experimental inoculation of hamsters leads to various kinds of neoplasms, depending primarily on the route of inoculation. Subcutaneous inoculation results in fibrosarcomas, hepatomas, or hepatic carcinomas, and intracranial inoculation leads to the development of ependymomas (1, 15). Quail bronchitis virus/CELO has not been found to be oncogenic in mice or chickens (15).

Pathogenesis and Epidemiology

Natural and Experimental Hosts

Bobwhite quail are the principal species that develop clinical signs and mortality due to infection with QBV. Clinical disease has been reported in Japanese quail. Chickens and turkeys may be experimentally infected but develop few or only mild clinical signs. Inapparent infections of chickens are suggested by serologic evidence (19, 20).

Transmission, Carriers, and Vectors

Quail bronchitis is highly contagious, as demonstrated by explosive morbidity and mortality in susceptible flocks. Most signs are

seen in quail younger than 6 week of age. Although not experimentally documented, transmission is probably by aerosol. However, fecal-oral or mechanical transmission has been documented for other avian adenovirus, and QBV has been isolated from cecal tonsil during experimental infections (12). Serologic evidence of infection of other gallinaceous birds may suggest that, even though they fail to develop clinical signs, these species may serve as a vector for QBV.

Incubation Period, Signs, Morbidity, and Mortality

Quail bronchitis is often a catastrophic disease of captive-reared bobwhite quail, which is manifested by respiratory distress that leads to death in young quail. Morbidity and mortality from field cases frequently exceed 50% and may be much higher in flocks affected at younger than 3 weeks of age. In experimental infections of 1-week-old quail, mortality began 2 days following intratracheal infection and subsided by day 9 (7). Mortality in quail inoculated at 3 weeks of age occurred between 6 and 11 days PI. Death is uncommon in birds older than 6 weeks of age.

Frequently, the first reported sign in the flock is a sudden increase in mortality. Closer inspection, however, frequently reveals that sick birds that demonstrate decreased feed consumption, ruffled feathers, huddling under brooders, wing droop, open-mouthed breathing, "snicks," and nasal-ocular discharge. Following infection, signs may develop at as early as 2 days but generally develop in 3–7 days. Severity of infection varies depending on the age at which the bird is infected. Quail bronchitis is most severe in quail less than 3 weeks of age. Older birds frequently are asymptomatic but develop antibodies to group I/serotype 1 adenovirus. This suggests that survivors may be immune to subsequent virus exposure, but the persistence of these antibodies and the level of immunity have not been investigated. Antibodies against QBV have been identified in recently hatched quail that did not exhibit adverse signs of infection. These antibodies were lost at between 4 and 6 weeks of age, suggesting that there were maternal antibodies.

Gross Lesions and Histopathology

The principal lesions of QB are in the respiratory tract (9). Nasal-ocular discharge may also be noted. Opacity and filling of the trachea by pale, moist, necrotic, and sometimes hemorrhagic exudate is common (Fig. 9.11A). On cross section, the mucosa is markedly thickened (Fig. 9.11B). Similar exudate may be found in the anterior air sacs. Histologically, tracheal lesions may include epithelial deciliation, cell swelling, karyomegaly, necrosis, desquamation, and leukocyte infiltration (Fig. 9.11C). Basophilic, intranuclear viral inclusions are common in intact or desquamated tracheal epithelium. Electron microscopic changes are similar to those seen histologically but also demonstrate phagocytosed viral particles.

In the lungs, red, consolidated areas surround the bronchial hilus (Fig. 9.11D). On section, bronchi frequently contain exudate similar to that in the trachea, indicative of a necrotizing, proliferative bronchitis. Inflammatory exudates consisting of lymphocytes, heterophils, and fluid may extend into the surrounding pulmonary parenchyma, but the intensity of the leukocyte response varies and may be confounded by secondary bacterial infections. Histologically, bronchial changes are similar to those in the trachea, except that bronchi may demonstrate more epithelial proliferation. Most lesions are associated with large basophilic intranuclear inclusions (Fig. 9.11E).

Lesions in the liver include multifocal pale, pinpoint to 3 mm necrotic foci. Histologically, these foci are characterized by hepatocellular necrosis, infiltrated to varying degrees by lymphocytes and fewer heterophils. Inclusion bodies are occasionally seen in hepatocytes adjacent to necrotic foci and/or biliary epithelium.

Lesions occur in the spleen and bursa of Fabricius but can be difficult to identify in quail less than 3 weeks of age. The spleen may be mottled and slightly enlarged. Histologically, affected spleens have multifocal, often extensive zones of necrosis, characterized by lymphocytolysis with increased fibrillar eosinophilic intercellular material, with minimal leukocyte infiltration. Adenoviral inclusions are rare in the spleen. Histologic lesions of the bursa of Fabricius include necrosis of lymphocytes, frequently accompanied by generalized lymphoid depletion and follicular atrophy. Intranuclear viral inclusions are common in bursal epithelium. Experimentally, some quail also develop necrotizing pancreatitis associated with adenoviral inclusions.

Immunity

The duration of immunity in QB is not known, but survivors of both naturally occurring and experimental infections were refractory to challenge with QBV for at least 6 months, and significant antibody levels developed in serum of quail following infection (2, 3, 16). Young chicks with maternal antibody are also refractory to challenge with QBV, but maternal antibody is not believed to prevent virus multiplication.

Diagnosis

In quail chicks, sudden onset of rales, sneezing, or coughing that spreads rapidly through the flock and results in mortality suggests QB. Excess mucus in the trachea, bronchi, and air sacs is added evidence of the disease. Severity of signs, rapidity of spread, and the presence of lesions are less marked in older quail. Isolation and identification of an agent indistinguishable from QBV (or CELO virus) would confirm the diagnosis. Inoculation of 9–11-day embryonating chicken eggs via the chorioallantoic sac with suspensions of trachea, air sacs, or lungs has been used for isolation of the virus. Yates *et al.* (23) recommended suspensions of fecal samples or homogenates of the posterior small intestine (ileum) or colon. Jack *et al*. (12, 13) reported good success in isolating QBV from the liver of naturally infected birds and from the bursa of Fabricius and cecal tonsils of experimentally infected birds. Three to five blind passages are made with allantoamnionic fluid harvested from chilled eggs up to 6 days or more PI or earlier from embryos that died 24 hours PI or later or that exhibit signs of stunting in daily candling. According to Yates *et al.* (23), a few strains seem to require inoculation via the yolk sac in 5–7-day embryos.

Embryo mortality (increasing with number of passages), stunting, thickening of the amnion, necrotic foci, or mottling of the liver, and accumulation of urates in the mesonephros are typical changes caused by QBV or CELO virus. Neutralization of the isolated virus by specific QBV or CELO virus antiserum would confirm identification of the virus and the diagnosis.

In general, information pertaining to isolation, propagation, and identification of CELO or any group I/serotype 1 avian adenovirus would be applicable to QBV. Yates *et al.* (23) noted preference for chick embryo kidney or kidney cell cultures, and Jack and Reed (10, 11) have described propagation of QBV in chicken embryo liver tissue. The agar gel precipitation (AGP) test may be used to place an isolated virus in the avian adenovirus group, but it does not identify the serotype. Serotype classification is based on virus neutralization (10). In the absence of virus isolation or with failure to isolate a virus, the AGP test, using stock antigen on paired sets of serum samples, may be of value. A markedly higher percentage of positive precipitin tests among samples collected during convalescence (2–4 weeks after initial signs) than among sera collected during the acute phase (first few days of signs) should add weight to a presumptive diagnosis of QB based on clinical observations.

Pulmonary aspergillosis may be differentiated from pox by the presence of caseous plugs in lungs or deposits in air sacs with pockets of grayish or greenish spore accumulations. Although bacterial infections might complicate the disease, none is known to cause rapid development of signs, lesions, and mortality typical of QB. DuBose (2) suggested that Newcastle disease might present a clinical picture similar in part to QB, but clinical Newcastle disease has not been described in bobwhite quail. Histologic identification of intranuclear inclusion bodies morphologically characteristic of adenovirus in tracheal or bronchial epithelium is highly suggestive of infection with QBV (9).

Treatment, Prevent, and Control

No specific treatment exists for QB. Increased warmth in the brooder house, adequate ventilation but no drafts, and avoidance of crowding are suggested supportive measures during an outbreak. Prevention is based on protecting susceptible quail from all possible sources of QBV or CELO virus. In addition to the usual sanitation procedures and measures to prevent entry of infectious agents onto the premises, care should be taken to keep adult quail, as well as other avian species, away from young quail. Control measures on a farm should be started immediately, when even a tentative diagnosis of QB has been made. In addition to general measures to prevent transmission from group to group, hatching operations may need to be deferred until 2 weeks after signs have disappeared in order to prevent an outbreak in the presence of highly susceptible young quail.

Attempted eradication of QBV from bobwhite quail on a large gamebird farm was unsuccessful but may have been responsible for preventing losses and clinical QB over a 2-year period (3). In that effort, 80% of the 10,000 quail hatched during the previous year died from the disease. In addition to measures described previously, older quail were marketed, and only survivors from hatches that had been affected at less than 4 weeks of age were kept for breeders. Virus-neutralization antibody at a high level was detected in 3-month-old quail hatched 2 years later, but no signs of QB were detected in the intervening period up to the time the farm closed the following winter. Winterfield and Dhillon (17) used a type 1 adenovirus serotype in quail chicks as a vaccine against QB. The isolate, designated Indiana C virus, was isolated from chickens (18). It proved nonpathogenic for quail in a laboratory trial and was subsequently used on a farm where QB was endemic and losses were extensive. It was reported that the disease quickly subsided. In recent studies (11), however, experimental inoculation of quail at 1 or 3 weeks of age resulted in mortality rates of 33–100%. In quail inoculated at 6 or 9 weeks of age, mortality ranged from 0–10%. Gross and histologic lesions included necrotizing tracheitis and bronchitis with pneumonia, necrotizing hepatitis and splenitis, and lymphoid depletion of the bursa of Fabricius. Based on these findings, Indiana C appears to be highly pathogenic for bobwhite quail and is not recommended for use as a vaccine to prevent QB. More studies on potential use of vaccines to prevent QB are needed.

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Chapter 10

Pox

Deoki N. Tripathy and Willie M. Reed

Introduction

Definition and Synonyms

Pox is a common viral disease of commercial poultry (chickens and turkeys) as well as of pet and wild birds. Of the approximately 9000 birds species, about 232 in 23 orders have been reported to have acquired a natural pox virus infection (11). Fowl pox is an economically important disease of commercial poultry because it can cause a drop in egg production and mortality. Synonyms that have been used for avipox infections are contagious epithelioma; avian diphtheria; variole aviaire [Fr.]; difteria aviar [Sp.]; bouba aviaria [Port.]; Geflugelpocken [Ger.]; virula aviar; and variola gallinarum. Pox is a slow-spreading disease characterized by the development of discrete nodular proliferative skin lesions on the nonfeathered parts of the body (cutaneous form) or fibrino-necrotic and proliferative lesions in the mucous membrane of the upper respiratory tract, mouth, and esophagus (diphtheritic form). A concurrent systemic infection may also occur.

Economic Significance

Mortality in flocks exhibiting the mild cutaneous form of the disease is usually low. However, it may become high with generalized infection, especially when lesions are primarily diphtheritic or when the disease is complicated by other infections or poor environmental conditions. The systemic form of disease in canaries causes high mortality.

Public Health Significance

Avian pox is not of public health significance. It does not cause productive infection in mammalian species. However, a pox virus isolated from a rhinoceros by Mayr and Mahnel (62) was characterized as fowl pox virus (FPV).

History

Pox has long been observed in several avian species. The term *fowl pox* initially included all pox virus infections of birds, but now it is primarily used to refer to the disease in commercial poultry. Initially, Woodruff and Goodpasture (146,147,148) presented evidence that the virus particles (Borrell bodies) within the inclusion bodies (Bollinger bodies) were the etiologic agent of fowl pox. Later, Ledingham and Aberd (57) demonstrated that antisera produced against FPV after immunization or following recovery from infection agglutinated a suspension of elementary bodies of FPV.

Etiology

Avian pox viruses (fowl, turkey, pigeon, canary, junco, mynah, psittacine, quail, sparrow, crow, peacock, penguin, alala, apapane, condor, and starling) are members of the genus *Avipoxvirus* of the family Poxviridae (74,130). FPV is the type species of the genus. Because of its economic importance, more basic and applied studies have been done on FPV than any other member of this genus.

Morphology

Like members of other genera of the Poxviridae family, all avian pox viruses have a similar morphology. The mature virus (elementary body) is brick shaped and measures about 330 × 280 × 200 nm. The outer coat is composed of random arrangements of surface tubules (Fig. 10.1A). FPV consists of an electron-dense centrally located biconcave core or nucleoid and two lateral bodies in each concavity and surrounded by an envelope (Fig. 10.1B).

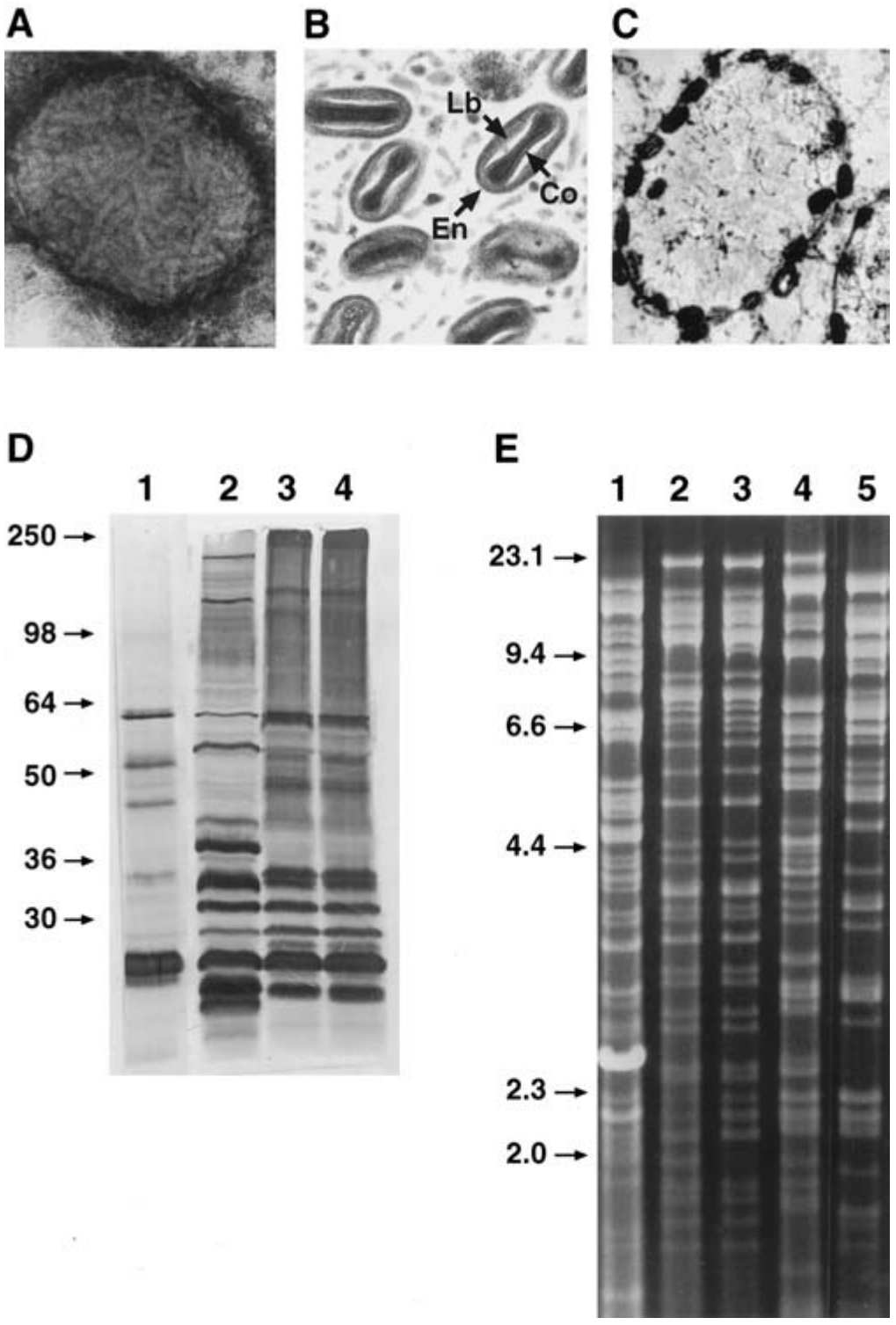
Chemical Composition

The main components of FPV are protein, DNA, and lipid. The virus has a particle weight of 2.04×10^{-14} g and contains 7.51×10^{-15} g protein, 4.03×10^{-16} g DNA, and 5.54×10^{-15} g lipid (84); nearly one-third of FPV is lipid. Squalene as a major lipid component and elevation of cholesterol esters were detected in virus preparation from infected chick scalp epithelium (60,141). The average weight of the inclusion body is about 6.1×10^{-7} mg, 50% of which is extractable lipids. The protein content per inclusion body is 7.69×10^{-8} mg, and the average weight of DNA per inclusion is 6.64×10^{-9} mg (83).

Virus Replication

The cytoplasmic site of DNA synthesis and packaging within the infectious virus particle is characteristic of pox viruses. Detailed information on replication of pox viruses may be found elsewhere (13, 18,73).

FPV contains genes that encode for a DNA ligase, ATP-GTP binding protein, uracil DNA glycosylase, DNA polymerase, DNA topoisomerase, DNA processing factor, and replication-essential protein kinase (1). In addition, FPV possesses a gene that encodes for the DNA repair enzyme, CPD photolyase that



10.1. A. Negatively stained FPV showing random distribution of surface tubules B. Ultrathin section of cutaneous pox lesion from a naturally infected dove showing virus particles of typical pox virus morphology. Co, core; Lb, lateral bodies; En, envelope (Basgall). C. Ultrathin section of diphtheritic fowl pox lesion from a chicken showing an A-type inclusion body in which virus particles of typical pox virus morphology are distributed around the periphery of the inclusion body. D. Strain variation in antigenic composition by immunoblotting of soluble antigens of avian pox viruses. Antigens prepared from cells infected with FPV strains: 101 (Lane 1) Ceva (Lane 2), Minnesota (Lane 3) and Nebraska (Lane 4). Proteins were separated by SDS-PAGE and transferred to nitrocellulose. Viral antigens were detected by reaction with chicken anti-FPV serum. E. Agarose gel electrophoretic analysis of the genomes of a field isolate of mynah pox (1), a vaccine and field isolate of fowl pox (2 and 3, respectively), a field isolate of canary pox (4), and a vaccine isolate of quail pox (5) virus after cleavage with HindIII. The scale on the lefthand side of the gel represents the position of the l HindIII fragments (kb).

repairs UV-induced damage to the DNA by using visible light as a source of energy. It is assumed that the presence of this enzyme helps the virus prolong its survival in external lesions in poultry and in the environment (107, 109).

Replication of avian pox viruses appears to be similar in dermal or follicular epithelium of chickens, ectodermal cells of the chorioallantoic membrane (CAM) of developing chicken embryos and embryonic skin cells. Differences in the host cell and virus strain, however, may be reflected in the time scale of replication and virus output.

Biosynthesis of FPV in dermal epithelium involves two distinct phases: a host response characterized by marked cellular hyperplasia during the first 72 hours and synthesis of infectious virus from 72–96 hours postinfection (PI) (20, 21).

The replication of viral DNA in dermal epithelium begins between 12 and 24 hours PI and is followed by the appearance of infectious virus later. Epithelial hyperplasia between 36–48 hours PI ends in a 2.5-fold increase in cell number by 72 hours PI. The rate of viral DNA synthesis is low during the first 60 hours of infection. Enhancement in the rate of viral DNA synthesis occurs between 60 and 72 hours PI concomitantly with a sharp decline of cellular DNA synthesis. Between 72 and 96 hours PI, the synthesis of viral DNA becomes progressively more prominent, and no further hyperplasia is observed (20, 21). Swallen (111) demonstrated by using autoradiography that chicken epidermis infected for 48 hours with FPV showed a three-fold increase in labeled nuclei as compared with controls, indicating that infection is associated with an increased incidence of intranuclear DNA synthesis. Based on positive hybridization signals, both viral RNA and DNA were detected in the nucleus of infected cells by 24–72 hours after infection (34).

Infection of chicken embryo skin cell culture results in an increase in virus titer at 16 hours after infection with evidence of cytopathic effects (CPE). Although the viral titer continues to increase during the next 20 hours, it then declines between 36 and 48 hours PI. A total increase in FPV titer of 100-fold is observed during the growth period. FPV DNA replication in these host cells occurs between 12 and 16 hours PI and continues through 48 hours PI (80).

The genome of FPV contains six genes with putative protein modification functions. These include three serine/threonine protein kinase (PK), one tyrosine PK, a metalloprotease, and a tyrosine/serine protein phosphatase. These are involved in phosphorylation of virus proteins during virion assembly, viral protein processing, and virion morphogenesis (1).

Based upon recent sequence analysis, FPV encodes homologues of at least 31 known vaccinia virus structural proteins (1), and the majority of them are associated with the intracellular mature virus particle (IMV). Of these proteins, 12 are located within the core, and 7 are associated with the membrane. Three proteins also are associated with extra-cellular enveloped virions (EEVs). In addition, homologues of 5 proteins, which represent 2 conserved pox virus gene families and have putative structural functions, are present in FPV. Also, FPV contains homologues of pox virus A-type inclusion (ATI) proteins. These inclusions protect mature virions from environmental insults and assist in prolonged survival in nature. Stability of the virus in the environment may be further supported by the presence of photolyase and glutathione peroxidase (1, 107).

Ultrastructural studies have focused on the morphogenesis of the virus in various developmental stages that lead to mature virions (4,5,87). After adsorption to and penetration of the cell membrane by FPV, and within 1 hour after infection of dermal epithelium (5) and 2 hours after infection of CAM (4), there is uncoating of the virus before synthesis of new virus from the precursor material. A few small virus factories containing crescents and a few isolated immature viruses (IV) are observed at 12 hours PI. The virus factories increase in size and contain more IV by 16 hours PI. Although between 16–66 hours PI, the majority of viral particles appear as IV, all subsequent steps of morphogenesis may also be observed. Only a few isolated intracellular mature viruses (IMV) are detected by 47 hours PI, some of which appear completely or partially wrapped and in the process of becoming intracellular enveloped virus (IEV). IMV particles are also found in clusters associated with membranes. Accumulation of viral particles near the plasma membrane suggests that FPV exits the cell mostly by budding (13). Inclusion bodies are present at 72 hours after infection of dermal epithelium (5) and at 96 hours after infection of the CAM (4). The A type inclusions may contain virions within or toward the periphery (Fig. 10.1C). Similar inclusions have been observed in cells infected with fowl, canary, and pigeonpox viruses and perhaps are made by all avian pox viruses.

Although pox viruses are assembled exclusively in the cytoplasm of infected cells, Gafford and Randall (34) found that the nucleus participates in the complexities of FPV replication, because viral RNA and DNA were detected in the nucleus of infected cells at 24–72 hours PI. Interestingly, an avian pox virus isolated from *Junco hyemalis* produced nuclear inclusions in addition to cytoplasmic inclusions (10). However, the intranuclear inclusions were devoid of viral particles.

Susceptibility to Chemical and Physical Agents

Resistance to ether treatment is listed as one of the taxonomic criterion for pox viruses (74). While some authors (84) stated that the virus was sensitive to both ether and chloroform, others (116) reported that a pigeon pox virus and two derived mutants were resistant to both chloroform and ether. FPV is known to withstand 1% phenol and 1:1000 formalin for 9 days but is inactivated by 1% caustic potash when freed from its matrix. Heating at 50°C for 30 minutes or 60°C for 8 minutes also inactivates the virus (3). Trypsin has no effect on the DNA or whole virus. When desiccated, the virus shows marked resistance. It can survive in dried scabs for months or even years.

Strain Classification

A nucleoprotein precipitinogen is common to all pox viruses (146). Avian pox viruses are antigenically and immunologically distinguishable from each other, but varying degrees of cross relationships do exist. Attempts have been made to differentiate strains by immunologic methods (e.g., complement-fixation, passive hemagglutination, agar gel precipitation, immunoperoxi-

dase, ELISA, virus neutralization, and immunofluorescence). Antigenic characterization of immunogenic proteins by immunoblotting (Fig. 10.1D) and genomic characterization by restriction fragment length polymorphism (RFLP) of DNA (Fig. 10.1E) have been useful to some extent in detecting minor differences among the strains tested (38, 77, 88, 101, 112). A monoclonal antibody against a unique antigen of FPV has been used to differentiate FPV strains (100,102). In recent years, FPVs have been isolated in all regions of the United States from previously vaccinated chicken flocks experiencing high mortality due to the diphtheritic form and/or cutaneous form of pox. Cross-protection studies revealed that some of these isolates have a limited immunologic relationship to strains of pox viruses used in commercial vaccines, indicating that currently available vaccines are not effective in providing adequate protective immunity against challenge with these "variant" pox viruses (31, 32, 101).

Polypeptides

Twenty-eight polypeptides were detected in purified FPV by Obijeski *et al*. (78). Likewise, Mockett *et al*. (67) observed about 30 structural polypeptides in FPV, most of which were immunogenic. Twenty-one FPV-coded polypeptides were resolved by ^{35}S methionine pulse labeling, and 57 major structural polypeptides were identified in purified FPV preparations (80). Several major and minor immunogenic polypeptides of FPV strains have been resolved by immunoblotting (77, 88, 100). Antigenic differences among the vaccine and field strains of FPV have been observed. Quail pox virus showed distinct antigenic differences from FPV based on immunoblotting, although some common proteins were also detected (38). A pox virus isolated from the spleen of a condor was genetically, antigenically, and biologically different from FPV (46). Antigenic profiles of avian pox viruses isolated from endangered Hawaiian forest birds also show differences from those of fowl pox virus (47, 48).

Genomic Differences in Avian pox Viruses

Like other pox viruses, the FPV genome is composed of a single linear double-stranded DNA molecule with a hairpin loop at each end. Although the overall genomic organization of FPV appears to be similar to that of other members of the Poxviridae family, some genomic rearrangement has occurred. The electrophoretic profiles of restriction enzyme-digested FPV and vaccinia virus DNAs are distinct (75,88). Although the DNAs of fowl, pigeon, and junco pox virus have similar genomic profiles, restriction endonuclease analysis profiles of quail, canary, and mynah pox virus DNAs reveal marked differences from those of FPV.

Two strains of avian pox viruses from endangered Hawaiian forest birds, 'alala pox isolated from Hawaiian crows (*Corvus hawaiiansis*) and apapane pox [isolated from an Apapane species (*Himatoine sanguinea*)] are genetically and biologically different from FPV (132). Similarly, avian pox virus strains isolated from endangered Hawaiian forest birds (palila and Hawaiian goose) are biologically different from fowl pox virus (48).

The FPV genome contains a central coding region and two identical, inverted terminal repeat (ITR) regions of 9,520 bp at both termini. The complete sequence of the genome of a vaccine-like strain of FPV has been determined. (1). It contains 288,539 bp, which is slightly smaller than some previous estimates and encodes for 260 putative genes of 60 to 1,949 amino acids in length. The genome of a tissue culture passaged FPV strain FP9 is approximately 260 kb in size (55). Based upon homologies with other viral or cellular genes, 101 open reading frames (ORFs) of FPV have been assigned similar or putative functions. The nucleotide composition of FPV is 69% A + T, which is uniformly distributed over the entire length of the genome. Six small regions with higher G + C content (50%) are located in the terminal genomic regions. Because of the presence of multiple and in some cases large gene families, the genome of FPV is larger than other completely sequenced pox virus genomes. In this regard, 32% of the FPV genome is composed of 31 genes in the ankyrin repeat family, 10 genes in the N1R/p28 family, and 6 genes in the B22R family. The B22R ORFs alone comprise 12% of the viral genome. Because fewer ankyrin genes were found in the genome of fowl pox virus after extensive passage in tissue culture, it is likely that in other avian pox viruses the number of ankyrin repeat genes may vary. As pox virus ankyrin repeat genes have been associated with host range functions, loss or disruption of many of these genes may be associated with the narrowing of host range (1). Interestingly, the genomes of field isolates causing outbreaks of fowl pox in vaccinated flocks contain an integrated, nearly intact provirus copy of reticuloendotheliosis virus (REV). In contrast, only a variable-length, REV long terminal repeat (LTR) remnants are present in the genome DNA of all FPV vaccine strains. These remnants are also retained, presumably after the loss of the REV provirus, by a minor proportion of each field strain population (35, 69, 104, 105, 113).

Although a ubiquitin gene has been identified and sequenced in the genome of an avian pox virus isolated from Hawaiian forest birds (92), only fragmented remains of this gene were observed in the FPV genome (1).

Analysis of the sequence of a 4.5 kb HindIII fragment of condorpox virus DNA with corresponding region of FPV genome showed differences. In FPV 11 ORFs are confined in this region including sequences related to REV integration. Condor pox virus, however, contains only 8 ORFs and does not have any REV sequences (46). Complete nucleotide sequence of canary pox virus has been determined (136). Nucleotide sequences of a 5.3 kb Pst-HindIII fragment of the genome of an avian pox virus from a Hawaiian goose (HGP) revealed very high homology with canarypox virus and homologs of three fowl pox virus ORFs including REV sequences were not present in its genome (47).

The complete nucleotide sequences of the genomes of other members of the avipox virus genus are not available at this time. However, based upon RFLP comparisons and nucleotide sequence analysis of selected genomic fragments, it is clear that genetically different strains of pox viruses infect domestic, pet, and wild birds. In this regard, the genomic profiles of canary pox, mynah pox, 'alala pox, apapane pox, condor pox, goose pox and quail pox viruses are markedly different from those of FPV (38, 46, 47, 88, 132). Moreover, the sequence analysis of the thymidine kinase (TK) gene of quail pox virus revealed only moderate homology to the corresponding FPV TK gene.

Nonessential and Immunomodulatory Genes

Based on the information available on vaccinia virus, it is clear that many of the genes of FPV may not be essential for virus replication in tissue culture, and some of them may be associated with immunomodulatory functions and may interfere with normal immune surveillance or host responses. The functions of the proteins encoded for by more than half of the genes of FPV are not known (1) and thus their role in virus replication is only speculative. Some putative genes and the probable functions of their encoded proteins are briefly described here: (a) A homologue of the eukaryotic transforming growth factor ß (TGFß), a multifunctional peptide that stimulates connective tissue growth and differentiation, is encoded by the genome of FPV. Because TGFß also exhibits a range of immunomodulatory effects, including suppression of cellular and humoral immune mechanisms, the FPV version may have a role in suppression of the host immune response and/or cell growth and differentiation. (b) Two ORFs encoding proteins similar to the cellular ß nerve growth factor (ß-NGF) have been identified in the FPV genome. These proteins may play some part in inhibiting antiviral immune responses in virus-infected cutaneous and respiratory tract infections. (c) Four ORFs of FPV show similarity to the CC class of small soluble chemokines. CC chemokines are known to attract T lymphocytes and NK cells to sites of infection. These CC chemokine homologues could function as antagonists and cause a broad-range inhibition of normal CC chemokine function during host antiviral immune responses. (d) Three genes encoding proteins homologous to G-protein-coupled receptors are present in the FPV genome. It is likely that the encoding proteins may bind to chemokines involving cell signaling that affect viral replication and pathogenesis in the host. (e) An ORF of FPV encodes a putative IL-18 binding protein. Because IL-18 homologues have been found to inhibit IL-18 dependent gamma interferon production, it may have an anti-inflammatory function during fowl pox virus infection. (f) An ORF of FPV with homology to semaphorins is likely to be associated with immunomodulatory function. (g) The FPV genome has eight ORFs that encode proteins similar to C-type lectins NKG2 and CD94 proteins present on NK cells and CD69 located on the surface of lymphocytes. C-type lectin cellular NK cell receptors bind class I major histocompatibility complex antigens and promote or inhibit immune activity through intracellular signaling pathways. It is likely that the expression of these proteins in FPV-infected cells interferes with normal immune surveillance or host responses. (h) Five homologues of serine proteinase inhibitors (serpins) encoded by FPV may be associated with host-range functions involving anti-inflammatory activity and/or regulation of cellular apoptosis in specific cells (1).

One of the characteristic features of avian pox virus infection is cellular hyperplasia of affected tissue. In this regard, a gene encoding a protein similar to epidermal growth factor (EGF) is present in the genome of FPV. Although this virus protein is not essential for virus replication, it may influence virulence, stimulate cell proliferation, and contribute to the hyperplasia observed in infected tissues. Further, a homologue of the T10 gene that encodes a protein expressed at high levels in epithelial cells in the trachea, esophagus, and lung of vertebrates is present in the genome of fowl pox. This T10 homologue may be required to extend the virus's host range to epithelial cells of the respiratory tract (1).

FPV DNA contains an ORF that encodes for a putative protein with similarity to the protein encoded by Marek's disease virus and fowl adenovirus indicating its role in avian host range function. Interestingly, a natural dual viral infection of trachea by FPV and herepesvirus has been reported previously (33). Because homologues of FPV open reading frames were detected in the genome of Marek's disease virus (17), the likelihood of exchange of genetic material from one virus to other and emergence of a different virus is possible. In this regard, integration of REV sequences in the FPV genome indicates "natural genetic engineering" in viruses. The FPV genome also contains a homologue of the glutathione peroxidase gene whose product may provide protection from oxidative stress, allowing efficient replication of virus under environmental conditions. Interaction of this enzyme and other proteins (e.g., photolyase) may have a synergistic effect on prolonging the survival of the virus in the poultry environment (1,107,109).

Hemagglutination (HA) activity has been detected in a few strains of pigeon pox virus (36,116) and in one strain of FPV (138). Although such HA activity is not present in most avian pox viruses, recently, the sequence of a putative HA gene has been identified in the genome of FPV (1). A similar nucleotide sequence is also present in the DNAs of other strains of FPV, but functional HA activity could not be demonstrated when using chicken red blood cells. The gene appears to be nonessential for virus replication in tissue culture, and its functional role is not known at this time.

Laboratory Host Systems

Birds

Avian pox viruses affect a wide range of birds of various families by naturally occurring or artificial infection. These viruses productively infect only avian species, indicating a significant degree of adaptation to the avian host. In this regard, a number of genes showing similarity to cellular genes have been found in the genome of FPV (1). Some of these (e.g., those encoding for TGb and b-NGF) may be involved in immune modulation.

A substantial degree of host specificity exists among some avian pox viruses, especially those that infect wild birds. A pox virus from a flicker (*Colaptes auratus*) (49) revealed strict host specificity when several species of wild and domestic birds were tested for susceptibility. Avian pox virus strains isolated from various species of thrushes (*Turdidae*) did not protect chickens against FPV (51). Differences in host susceptibility were also observed when a pox virus isolated from parrots was inoculated into susceptible parrots and chickens. Although it was more pathogenic for parrots than chickens, it did not provide protection against FPV. Further, vaccination of chickens with either fowl or pigeon pox virus vaccine did not provide protection against challenge with psittacine pox virus (12). A pox virus from a Canada goose (*Branta canadensis*) could be transmitted to domestic geese but not to chickens or domestic ducks (25).

Sparrows and canaries were highly susceptible to a pox virus isolated during an outbreak in sparrows but produced a mild, local cutaneous reaction in chickens, turkeys, and pigeons (39). Chickens and pigeons were found to be refractory to infection with an avian pox virus isolated from a buzzard (*Accipiter nisus*) (117). In an aviary housing more than 100 birds of a variety of species, only Rothchild's mynahs (*Leucospar rothchildii*) were infected with an avian pox virus. The virus, however, was pathogenic for starlings in the surrounding area but did not infect chickens. Mynahs and starlings are members of the family Sturnidae, and starling pox has been reported to be specific for birds in that family (56). Pox virus strains isolated from magpies (*Pica pica*) and great tits (*Parus major*) did not infect young chickens (42). Pox virus strains from various species of grouse immunized chickens against a FPV challenge (50). An isolate of pox virus from previously vaccinated turkeys was antigenically different from FPV (145). Pox virus isolated from cutaneous proliferative lesions of greater hill mynah (*Gracula religiosa*) imported from Malaysia produced severe necrotizing and proliferative lesions in chickens and bobwhite quail previously vaccinated with fowl, pigeon, or quail pox viruses (85, 86). In an outbreak of avian pox, canaries and house sparrows were affected, although 10 species of passerine birds were housed within the facility (27).

Avian pox virus infections have been considered to be important factors in limiting the population of Hawaiian forest birds. Three isolates of pox viruses from Hawaiian forest birds (alala and apapane species) produced mild lesions in chickens, and only two of them could be adapted to chicken cell lines. A comparison of their genomic profile by RFLP revealed that the two Hawaiian bird pox viruses appeared to be more related to each other than to FPV, indicating that genetically distinct pox viruses exist in Hawaiian forest birds (132). Two avian pox viruses from endangered Hawaiian forest birds (Hawaiian goose and Palila) produced only a localized lesion in susceptible chickens. The lesion persisted for a short duration and the birds were not protected against challenge with fowl pox virus (48)

Studies on the differentiation of fowl, canary, turkey, and pigeon pox viruses based on pathogenicity for chickens, turkeys, pigeons, ducks, and canaries (26, 37, 61) have been summarized. Canaries are highly susceptible to canary pox virus but show resistance to turkey, fowl, and pigeon pox viruses. Pigeon pox virus produces a mild infection in chickens and turkeys but is very pathogenic for pigeons. Susceptibility of ducks to turkey pox virus and not to FPV has been suggested for differentiation of these two closely related viruses.

Avian Embryos

Developing chicken embryos from a specific-pathogen-free supply (SPF) are commonly used for initial isolation and propagation of avian pox virus by inoculation of the CAM (93, 129, 130, 147). Ten–twelve day-old developing chicken embryos are appropriate for virus isolation. Duck and turkey embryos have been used, as well as embryos of other avian species. The inoculated embryos are incubated at 37°C for 5–7 days. Typically, infection of chicken embryo CAM results in compact, proliferative pock lesions that may be focal or diffuse (Fig. 10.2D). Quantitative assay of viral infectivity may be performed by using the embryo infective dose-50% (EID_{50}) method or by the "pock counting enumerative dose" response (139). Occasionally, isolates from wild birds fail to grow on the chorioallantoic membrane CAM of chicken embryos.

Cell Culture

Avian pox viruses can be propagated in cell cultures of avian origin (e.g., chicken embryo fibroblasts, chicken embryo dermis and kidney cells, and duck embryo fibroblasts). A permanent cell line "QT 35" (88) of Japanese quail origin as well as the chicken liver cell line LMH (44) will support growth of some avian pox viruses after adaptation. However, some isolates, especially from turkeys and wild birds, fail to grow in these cell lines even after repeated passages. While avian pox virus infections of mammalian cells are believed to be abortive, in a recent study (141) Syrian baby hamster kidney (BHK-21) cells were found permissive for three avian pox virus strains.

Cytopathic Effects

Characteristic CPE produced by the avian pox viruses in chicken embryo fibroblasts and QT 35 cells is characterized by an initial phase of rounding of the cells followed by a second phase of degeneration and necrosis. Quantitative assay is by the cell culture dose-50% method based on CPE (139).

Plaque Formation

Differences in the plaque-forming ability of avian pox viruses have been observed. Adaptation of the virus in cell culture is necessary, because not all strains produce plaques (71). Plaque formation in monolayers of chicken embryo fibroblast cell cultures by some avian pox viruses was shown to be characteristic and is considered as an aid in differentiation (61). Plaques are evident by 3–4 days PI in quail cells with certain avian pox viruses after adaptation (88).

Pathobiology and Epidemiology

Incidence and Distribution

Avian pox viruses infect birds of both sexes and all ages and breeds. The disease has been reported in more than 200 avian species (11). Fowlpox, in commercial poultry, is worldwide in distribution (79). The incidence, however, is variable. In high-density areas where multiple age birds are raised under confined conditions, the disease tends to persist for a long time despite preventive vaccinations. In recent years, several outbreaks of the diphtheritic form of fowl pox have been encountered in previously vaccinated chicken flocks.

Natural and Experimental Hosts

Fowl and turkey pox virus infections are economically important diseases in domestic poultry. Among companion birds, avian pox virus infections most often occur in blue-fronted Amazon parrots and in large aviaries of canaries where the disease is likely to be enzootic because of intimate contact. Canary pox and psittacine

pox are, therefore, of special significance for aviculturists, as the disease can result in high losses in a short time. Severe outbreaks of quail pox in pen-raised quails have been reported.

Because of convenience, reasonable cost, and ready availability, susceptible chickens of various ages are used widely as experimental hosts in biological characterization of avian pox virus isolates. Most of the pathogenesis studies, however, have been conducted with FPV. Pathogenesis of FPV infection, in chickens inoculated intradermally or intratracheally, was similar with only minor differences. In chickens infected intradermally, the virus was first detected in the skin at the inoculation site on day 2 and in lungs on day 4, followed by detectable viremia on day 5 PI. In chickens infected intratracheally, the virus was first detected in the lungs on day 2, followed by viremia on day 4 PI. The virus was recovered from the liver, spleen, kidney, and brain of birds of both groups (99). In chickens inoculated intravenously, miliary nodules were observed in the kidneys at 10–18 days PI in addition to cutaneous lesions and diphtheritic lesions on the mucous membrane of the upper respiratory tract. Characteristic microscopic changes including inclusion bodies were observed in the epithelial cells of renal tubules 4–14 days PI, and in the epithelial reticular cells of the thymic medulla 4–10 days PI (115).

Transmission

Pox virus infection occurs through mechanical transmission of the virus to the injured or lacerated skin. Individuals handling birds at the time of vaccination may carry the virus on their hands and clothing and may unknowingly deposit the virus in the eyes of susceptible birds. Insects also serve as mechanical vectors of the virus, resulting in ocular infection. The virus may reach the laryngeal region via the lacrimal duct to cause infection of the upper respiratory tract (30). In a contaminated environment, the aerosol generated by feathers and dried scabs containing pox virus particles provide suitable conditions for both cutaneous and respiratory infection. Cells of the mucosa of the upper respiratory tract and mouth appear to be highly susceptible to the virus as initiation of infection may occur in the absence of apparent trauma or injury. Detection of lesions only in the lungs in the absence of other afflicted areas is suggestive of aerosol infection (64). A homologue of T10, which is specifically expressed at high levels in epithelial cells of the respiratory tract, is encoded by a gene present in the genome of FPV. Its presence may be related to host-range function.

Mosquitoes can infect a number of different birds after a single feeding on a bird infected with avian pox virus. Eleven species of Diptera have been reported as vectors of avian pox virus (2). The mite, *Dermanyss gallinae,* has been implicated in the spread of FPV (96). Mechanical transmission of FPV from infected toms to turkey hens through artificial insemination has been reported (65).

In some flocks, the virus may persist for extended periods. This is common in large multiple age complexes. Kirmse (49) observed persistent cutaneous lesions of avian pox virus infection in a yellow-shafted flicker over a period of 13 months during which intracytoplasmic inclusions were demonstrable in the lesion. In an experimental study, Duran-Reynals and Bryan (28) showed that cutaneous treatment of chickens and pigeons with methylcholanthrene activated a latent FPV infection.

Incubation Period

Incubation period of the naturally occurring disease varies from about 4–10 days in chickens, turkeys, and pigeons and is about 4 days in canaries.

Clinical Signs

The disease may occur in one of the two forms, cutaneous or diphtheritic, or both. In addition, a systemic form of infection with high mortality is usually seen in canaries. The signs vary depending upon the susceptibility of the host, virulence of the virus, distribution of the lesions, and other complicating factors. The cutaneous form of the disease is characterized by the appearance of nodular lesions on the comb, wattle, eyelids, and other nonfeathered areas of the body (Fig. 10.2A, C). Cutaneous eye lesions will interfere with the bird's ability to reach food and water. In the diphtheritic form (wet pox), cankers or diphtheritic yellowish lesions (Fig. 102B) occur on the mucous membranes of the mouth, esophagus, or trachea with accompanying coryza-like mild or severe respiratory signs similar to those caused by infectious laryngotracheitis virus infection of the trachea. Lesions in the corner of the mouth, on the tongue, throat, and upper part of the trachea interfere with eating, drinking, and breathing. In pullets coming into lay and in older birds, the disease often runs a slow course accompanied by unthriftiness and reduced egg production.

Morbidity and Mortality

Morbidity rate of pox in chickens and turkeys varies from a few birds being infected to involvement of the entire flock if a virulent virus is present and no control measures are taken. Birds affected with the cutaneous form of the disease are more likely to recover than those with the diphtheritic form involving oral mucosa and the respiratory tract.

Effects of pox in chickens usually involve emaciation and poor weight gain; egg production is temporarily retarded if layers are infected. The course of the mild cutaneous form of disease is about 3–4 weeks, but if complications are present, duration may be considerably longer. With virulent strains of fowl pox virus, both primary and secondary cutaneous lesions may persist for more than 4 weeks. In such cases, cutaneous lesions around the eyes or diphtheritc lesions in the mouth and upper respiratory tract interfere with normal functions resulting in significant mortality.

In turkeys, the retardation of growth development of market birds is of greater financial importance than mortality. Blindness due to cutaneous eye lesions and starvation cause most of the losses. If pox occurs in breeding birds, decreased egg production and impaired fertility may result. In uncomplicated mild infections, the course of the disease in a flock may be 2–3 weeks. Severe outbreaks often last 6, 7, or even 8 weeks.

Flock mortality in chickens and turkeys is usually low, but in severe cases it may be high. In pigeons and psittacines, morbidity and mortality rates are similar to those in chickens. Pox in canaries can cause mortality as high as 80–100%. Significant mortality has been observed in quail infected with quail pox virus. Pox virus infections have been considered as a population limiting factor for Hawaiian forest birds.

Pathology

Gross

The characteristic lesion of the cutaneous form of pox in chickens is a local epithelial hyperplasia involving epidermis and underlying feather follicles, with formation of nodules that first appear as small white foci and then rapidly increase in size and become yellow. In chickens infected intradermally, a few primary lesions appear by the fourth day. Papules are formed by the fifth or sixth day. This is followed by the vesicular stage, with formation of extensive thick lesions (66). Adjoining lesions may coalesce and become rough and gray or dark brown. After about 2 weeks or sometimes sooner, lesions have areas of inflammation at the base and become hemorrhagic. Formation of a scab, which may last for another 1–2 weeks, ends with desquamation of the degenerated epithelial layer. If the scab is removed early in its development, there is a moist, seropurulent exudate underneath covering a hemorrhagic granulating surface. When the scab drops off naturally, a smooth scar may be present; in mild cases, there may be no noticeable scar. Attenuated vaccine viruses produce localized lesions, which are mild in comparison to the severe ones due to the pathogenic strains. The secondary lesions produced by pathogenic strains may persist for several weeks (124)

In the diphtheritic form, slightly elevated, white opaque nodules or yellowish patches develop on the mucous membranes of mouth, esophagus, tongue, or upper trachea. Nodules rapidly increase in size and often coalesce to become a yellow, cheesy, necrotic, pseudodiphtheritic, or diphtheritic membrane (Fig. 10.2B). If the membranes are removed, they leave bleeding erosions. The inflammatory process may extend into sinuses, particularly the infraorbital sinus (resulting in swelling) and also into the pharynx and larynx (resulting in respiratory disturbances) and esophagus. It is not uncommon to find cutaneous infection characterized by the development of lesions on the comb and wattles as well as diphtheritic lesions in the mouth and/or respiratory tract of the same bird. Often involvement of the eyes and eyelids may accompany the formation of lesions in other areas of skin as well as diphtheritic lesions.

Although in some cases, avian pox virus infection may be characterized by cutaneous, diphtheritic, systemic and oncogenic manifestations (135), in others the infection may be localized and characterized by the presence of small pale firm nodules in some internal organs (64). In a natural pox virus infection in Galapagos doves (*Nesopelia g. galapagoensis*) small (1–6 mm), pale firm nodules in the lungs were characterized by lobulated and nonlobulated nodular foci, located mainly in the airways, originating from primary and secondary bronchi. The etiologic agent, pox virus particles were detected by electron microscopy (64).

In a dead 3-month old fledging Andean condor no lesions were found on the entire skin. However, the oral cavity, esophagus and crop had multifocal raised yellow plaques. Most internal organs including heart lungs, liver, kidney, small intestine, pancreas and spleen had single to multiple soft white nodules ranging in size from 0.2 to 0.8 cm in diameter. Cytoplasmic inclusion bodies were observed on histopathological examination of tissues. Also, particles with distinct pox virus morphology were observed in ultrathin sections of the oral cavity, spleen and liver when viewed by transmission electron microscopy (46).

The first indication of pox in turkeys is appearance of minute yellowish eruptions on the dewlap, snood, and other head parts. They are soft and easily removed in this pustular stage, leaving an inflamed area covered with a sticky serous exudate. The corners of the mouth, eyelids, and oral membranes are commonly affected. Lesions enlarge and become covered with a dry scab or a yellow-red or brown wartlike mass. In young poults, the head, legs, and feet may be completely covered with lesions. The disease may even spread to the feathered parts of the body. In an unusual outbreak of pox virus in breeding turkeys, proliferative lesions occurred in the oviduct, cloaca, and skin surrounding the vent (65).

Microscopic

The most important feature of infection (whether the lesion is cutaneous, diphtheritic, or from infected CAM) is hyperplasia of the epithelium and enlargement of cells, with associated inflammatory changes. Expression of an epidermal growth factor (EGF)-like domain present in the genome of FPV perhaps plays an important role in hyperplasia observed in FPV-infected tissues. Characteristic eosinophilic A-type cytoplasmic inclusion bodies (Bollinger bodies) are observable by light microscopy (Fig. 10.2E) in infected cells. Because pox viruses are the largest among viruses, the elementary bodies can be observed in smears prepared from the lesions and stained by the Gimenez method (126).

Histopathologic changes of tracheal mucosa include initial hypertrophy and hyperplasia of mucus-producing cells, with subsequent enlargement of epithelial cells that contain eosinophilic cytoplasmic inclusion bodies (Fig. 10.2E). These inclusion bodies appear green after staining with acridine orange, indicating DNA in the inclusions (Fig. 10.2F). Inclusion bodies may be present in various stages of development, depending on the time after infection and may occupy almost the entire cytoplasm, with resulting cell degeneration. Often, clusters of epithelial cells resembling a papilloma (114) may be observed.

Ultrastructural

Ultrastructural features of avian pox viruses are briefly described in the virus replication and diagnostic sections. Because of their large size, typical morphology, and characteristic ultrastructural details, diagnosis of avian pox viruses is relatively easy under electron microscopy by negative staining or in ultrathin sections (Fig. 10.1A, B, C).

Diagnosis

Diagnosis of avian pox virus infections is also described elsewhere (26, 127, 130, 133, 134).

Microscopy

Elementary bodies (Borrel bodies) of fowl pox virus can be detected in smears prepared from lesions, which are stained with Wright's stain or by the Gimenez method (126). Tissue sections from cutaneous or diphtheritic lesions may be processed by con-

ventional methods or by using a solution that fixes and dehydrates the tissues simultaneously (94) for detection of cytoplasmic inclusions (Fig. 10.2E). Various histochemical and histopathologic techniques have been described by Thompson and Hunt (121). Cutaneous lesions typical of avian pox (Fig. 10.2A, C) and diphtheritic lesions in upper respiratory and oral mucosa (Fig. 10.2B) must be confirmed by either histopathology (presence of cytoplasmic inclusions as shown in Fig 10.2E) or virus isolation.

Electron microscopy can be used for the demonstration of virus particles in lesions and exudate by negative staining or in ultrathin sections (Fig. 10.1A, B, C) of the infected tissues (22, 87). Type A inclusions with virions around the periphery or virus-filled inclusions may be observed during electron microscopic examination (Fig. 10.1C).

Isolation and Identification of Virus

Bird Inoculation

Avian pox viruses can be transmitted to susceptible birds by applying a suspension of the lesion material from infected birds to their scarified comb or denuded feather follicles of the thigh, or by the wing-web stick method. FPV can be transmitted readily from chicken to chicken, with typical cutaneous lesions developing in 5–7 days. In atypical cases, microscopy of lesion specimens, as well as bird inoculation, may be advisable.

Avian Embryo Inoculation

A suspension of a pox virus suspected specimen from a dermal or diphtheritic lesion is inoculated on the CAM of 9–12-day-old developing chicken embryos from a SPF flock; 5–7 days after inoculation, the CAM is examined for pock lesions (see Fig. 10.2D). Occasionally, some isolates fail to grow on the CAM of chicken embryos (25, 49).

Cell Culture

Cell cultures generally are not employed for initial isolation of avian pox viruses. Adaptation of the virus to this host system is sometimes necessary, because not all strains produce CPE on initial inoculation. For antigenic and genetic characterization of an isolate, propagation in cell culture is more convenient than the use of CAM.

Serology and Protection Tests

Actively acquired immunity against avian pox viruses results after recovery from naturally occurring infection or following vaccination. Both cell-mediated and humoral immunity following vaccination or naturally occurring infection provides protection (70, 125). Cell-mediated immunity develops earlier than the humoral antibody response. A lymphoproliferative response was observed following inoculation of chickens with fowl pox vius strains (103).

Immune responses against avian pox viruses can be determined by serological tests, such as ELISA, virus neutralization, or protection tests. Protection tests generally are used to determine immunogenicity of fowl and pigeon pox vaccines. For this purpose, at least 20 SPF chickens are vaccinated according to manufacturer's directions. An additional 20 nonvaccinated and isolated birds of the same source and age are kept as controls. At 3 weeks after vaccination, vaccinated and control birds are challenged with a different strain of FPV capable of causing clinical signs of pox in the control birds. The challenge virus may be applied to the skin of denuded feather follicles of the thigh, to scarified comb, or by the wing-web method at a site opposite to that used for vaccination. The birds should be examined for takes (see Immunization). For satisfactory immunization, at least 90% of the controls should have lesions of fowlpox and at least 90% of the vaccinated birds should not.

Cross-protection tests for the antigenic relationship of the avian pox viruses generally are not practical for routine diagnosis but may be necessary for their biological characterization (16, 48, 86, 101, 144).

Immunodiffusion

Immunodiffusion has been used for the identification of fowl pox and pigeon pox viruses and also to differentiate antibody responses due to fowl pox and from those of other avian viral diseases (43, 137). Although the test is easy and simple to perform, its sensitivity is low and because of cross-reacting antigens differential diagnosis may not be easy. As precipitating antibodies are detectable for only a short duration after infection, serum must be collected at the appropriate time, usually within 15–20 days after onset of infection.

Passive Hemagglutination

A passive hemagglutination test will detect antibodies in the serum of FPV infected chickens earlier than the immunodiffusion test (122). Although this test is very sensitive, its use has been limited because it requires sheep or horse red blood cells for sensitization with soluble pox virus antigens. Further, differentiation of viruses is not possible because of cross-reacting antigens.

Neutralization

Virus neutralization in cell culture (72) or chicken embryos may be used; however, this procedure is not convenient as a routine diagnostic test.

Fluorescent Antibody, Immunoperoxidase, and Enzyme-Linked Immunosorbent Assay (ELISA)

Direct or indirect immunofluorescence or immunoperoxidase tests will reveal specific staining of intracytoplasmic inclusions in virus-infected cells. In the indirect test, first, the antibody against FPV is incubated with the antigen present in the infected cells and then the bound antibody is reacted with a secondary fluorescein-isothiocynate-labeled or peroxidase-labeled antibody against chicken gamma globulin (e.g., goat anti-chicken) (19, 123).

Currently, ELISA is the test of choice to determine humoral antibody responses. An antibody response is detectable in 7–10 days PI.

Immunoblotting

Immunogenic proteins of vaccine and field strains of fowl pox virus can be compared by immunoblotting. Although common

antigens are detected (Fig. 10.1D), strains can be differentiated to some extent by the presence of unique proteins of differing electrophoretic mobilities (38, 77, 88, 97). Recently, two monoclonal antibodies have been used to characterize field isolates and vaccine strains of FPV (100, 102).

Molecular Methods

Molecular techniques for diagnosis of pox virus infections have also been summarized elsewhere (134).

Restriction Endonuclease Analysis of Avian Pox virus DNA. Restriction fragment length polymorphism (RFLP) can be used for comparing the genomes of avian pox viruses by examination of the relative mobilities of restriction endonuclease generated fragments of their DNAs (24, 88, 112). The genetic profiles of FPV strains are similar, with a high proportion of comigrating fragments, although most strains could still be distinguished by the presence or absence of one or two DNA fragments (Fig. 10.1E). The characteristic electrophoretic profile of restriction endonuclease digested DNA has facilitated comparison of other members of the *Avipoxvirus* genus. In this regard, genomic profiles of fowl pox, quail, canary, and mynah pox viruses are distinct (91). Similarly, the Hawaiian bird pox viruses, ‘alala pox and apapane pox, have genetic differences that distinguish each other as well as from FPV (46, 47, 132).

Genomic Fragments as Diagnostic Probes. Selected genomic fragments or oligonucleotides (89) designed from the published sequences of FPV have been used as probes in detecting FPV-specific DNA in the test samples. Crude DNA isolated from the skin or diphtheritic lesion is transferred to a solid surface (e.g., nitrocellulose membrane) and then hybridized with either a cloned fragment or an oligonucleotide, which has been radioactively labeled (usually with ^{32}P dCTP) or with a nonradioactive substance (e.g., digoxigenin). This procedure is sensitive and specific and can be used in mixed infections. For example, using virus-specific genomic probes, a dual fowl pox and infectious laryngotyracheitis virus infection was confirmed (33).

Polymerase Chain Reaction. Genomic FPV DNA sequences of various sizes can be amplified by polymerase chain reaction (PCR) using specific primers (45, 58, 59, 82, 106, 112). This technique is useful when an extremely small amount of virus is present in the sample. In case of mixed infections, fragments of different sizes can be amplified in a single PCR reaction using pathogen-specific primers. For example, the diphtheritic form of fowl pox and infectious laryngotracheitis produce similar clinical signs and tracheal lesions. Using virus-specific primers, either of these infections could be detected (52). Currently, PCR is being used to differentiate vaccine and field strains of FPV because the latter contain intact REV provirus, and the vaccine strains contain only REV LTR sequences (45, 101, 104, 112).

Differential Diagnosis

Because similar tracheal lesions in chickens can be produced by fowl pox and infectious laryngotracheitis virus, the diphtheritic form of fowl pox in chickens with associated respiratory signs must be differentiated from infectious laryngotracheitis. In the case of infectious laryngotracheitis, intranuclear inclusions are detected in the tracheal epithelium.

Lesions caused by pantothenic acid or biotin deficiency in young chicks (6) or by T-2 toxin (23, 151) could be mistaken for pox lesions. Similarly, diphtheritic pox lesions in doves and pigeons may be mistaken for lesions caused by *Trichomonas gallinae,* which are diagnosed by microscopic examination of smears or by culture.

Intervention Strategies

Management Procedures

Because of its genetic make-up and inherent stability, FPV can persist in scabs in the poultry environment and become a source of infection for the susceptible replacement young birds. The greater frequency of the disease is perhaps due to closer confinement of chickens, especially in multiple age large complexes. Such conditions provide opportunity for the transmission of the disease from bird to bird as well as through aerosol. Close confinement and unclean houses increase the opportunity for spreading the disease.

Immunization

Live virus vaccines are used for immunization of birds against pox. Vaccines of fowl pox and pigeon pox virus origin are routinely used for vaccination of chickens and turkeys in areas where the disease is endemic. These should contain a minimum concentration of 10^5 EID_{50}/ml (37, 143) to establish satisfactory takes for good immunity. Fowl pox and pigeon pox virus vaccines labeled “chick embryo origin” are prepared from infected CAM. FPV vaccine labeled “tissue culture origin” is prepared from infected chicken embryo fibroblast cultures.

The success of a vaccination program depends on the potency and purity of the vaccine and its application under conditions for which it is specifically intended. Vaccination essentially produces a mild form of the disease. Directions for use of vaccine as supplied by the producer should be followed explicitly. Vaccine should not be used in a flock affected with other diseases or in generally poor condition. All birds within a house should be vaccinated on the same day. Other susceptible birds on the premises should be isolated from those being vaccinated. If pox appears in a flock in an initial outbreak with only a few birds being affected, nonaffected birds should be vaccinated.

A vaccine vial should be opened immediately before use. Only one vial should be opened at a time, and the entire contents should be used within 2 hours. After vaccine is prepared, the vaccinator’s hands should be washed thoroughly. Vaccine should contact the bird only at the site of immunization. Extreme precautions should be taken not to contaminate other parts of the bird, the premises, or miscellaneous equipment.

All contaminated vaccine equipment, unused vaccine, empty vials, etc., should be decontaminated, preferably by incineration. No prepared vaccine should be saved for later use.

Fowl Pox Vaccine

The "chick embryo origin" vaccine contains live FPV capable of producing serious disease in a flock if used improperly.

Fowl pox vaccine is commonly applied by the wing-web method to 4-week-old chickens and to pullets about 1–2 months before egg production is expected to start. It is also used to revaccinate chickens held for the second year of egg production. The vaccine is not to be used on hens while they are laying.

Attenuated FPV vaccines of cell culture origin can be used effectively on chicks as young as 1 day of age and have been used at times in combination with Marek's disease vaccine (29, 98).

Oral vaccination with an attenuated cell culture vaccine was reported to be effective in Germany by Mayr and Danner (63). Successful immunization required 10^6 to 10^8 $TCID_{50}$ depending upon the vaccine virus used. Comparative immunity of FPV vaccines by intramuscular, feather follicle, oral, and intranasal routes in chickens of different age groups was evaluated by Sharma and Sharma (95). They reported that oral vaccination did not provide protection over 50%, and the other methods provided 80–100% protection. Nagy *et al*. (76) demonstrated that 1-day-old chicks can be vaccinated effectively against fowlpox through drinking water when the vaccine contains a sufficiently high concentration of virus (10^6 cell culture infective $dose_{50}$ per ml).

Turkeys may be vaccinated by the wing-web method, but the virus may spread and infect the head region. The site of choice for vaccination is about midway on the thigh. Initially, turkeys are vaccinated when they are 2–3 months old, but those to be used as breeders should be revaccinated before production. Revaccination at 3–4-month intervals during the laying season might be of some advantage, depending on the level of risk. Fowlpox vaccine is not to be used on pigeons.

In recent years, several outbreaks of fowl pox have occurred in all regions of the United States in chickens that had been vaccinated with either fowl pox or pigeon pox virus vaccines, indicating their inability to provide adequate immunity (31, 32, 101). Often combined fowl pox and pigeon pox virus vaccines have been used in chicken flocks with variable results. In this regard, field isolates of FPV from vaccinated flocks show variable pathogenicity in chickens. Most of the field strains contain full-length REV in their genome. Experimental studies indicate that FPV containing integrated REV provirus induces profound, but selective immunosuppressive effects on infected chickens of younger age (140). A comparison *in vivo* of a field strain of FPV, its genetically modified progeny (in which all REV sequences were deleted) and a rescue mutant (in whose genome the REV provirus was inserted in its previous location) indicated that elimination of the provirus sequences correlated with reduced virulence (105)

Pigeon Pox Vaccine

Pigeon pox vaccine contains live, nonattenuated, naturally occurring virus from pigeons. If used improperly, the vaccine can cause a severe reaction in these birds. The virus is less pathogenic for chickens and turkeys.

Pigeon pox vaccine may be applied by the wing-web method and can be used on chickens of any age. It is generally applied to chickens at 4 weeks of age and about 1 month before egg production is expected to start. When birds younger than 4 weeks are vaccinated, they should be revaccinated before the start of production. Birds held for the second year of production should be revaccinated.

Turkeys can be vaccinated at any age by the wing-web or thigh stick methods. Day-old poults can be vaccinated if necessary, but it is better to wait until they are about 8 weeks old so that a better immune response is obtained. Revaccination may be necessary and advisable during the growing period. Turkeys retained as breeders should be revaccinated.

Pigeons can be vaccinated by the wing-web method. The vaccine can be applied by the feather follicle method, but this is not generally employed. Differences in the immunizing properties of pigeon pox vaccines have been observed (150).

Canary Pox Vaccine

A live chicken embryo-attenuated canary pox virus vaccine has been used effectively in canaries under experimental conditions (40). A modified live canary pox virus vaccine to be administered cutaneously by the wing-web method is currently available commercially in the United States. Vaccination of birds at weaning age is suggested, and booster vaccinations are recommended every 6–12 months and 4 weeks prior to laying or vector season.

Quail Pox Vaccine

A live vaccine of quailpox virus origin is available commercially. It does not appear to provide adequate protection against FPV infection (32,144).

Turkey Pox Vaccine

A live nonattenuated vaccine is commercially available for use in turkeys. The vaccine does not provide adequate protection against fowl, pigeon, or quail pox viruses (145).

Vaccine Takes

The flock should be examined about 7–10 days after vaccination for evidence of "takes." A "take" consists of swelling of the skin or a scab at the site where the vaccine was applied and is evidence of successful vaccination. Immunity will normally develop in 10–14 days after vaccination. If the vaccine is properly applied to susceptible birds, the majority of the birds should have takes. In large flocks, at least 10% of the birds should be examined for takes. The lack of a take could be the result of vaccine being applied to an immune bird, use of a vaccine of inadequate potency (after the expiration date or subjected to deleterious influences), or improper application.

Prophylactic Vaccination

Immunization against pox consists of vaccinating susceptible birds prior to the time the disease is likely to occur. Usually this is done during spring and summer in areas where the disease occurs in fall and winter. However, in large complexes containing multiple age birds and in tropical climates, where the disease may occur throughout the year, vaccination may be performed at any time when warranted without regard to the season.

Vaccination against FPV is indicated under three conditions: a) When a flock on the premises was infected the previous year, all young stock produced on the premises or introduced from other sources should receive fowl pox vaccine. b) If pox was present the previous year and pigeon pox vaccine was used, birds should be revaccinated with fowl pox vaccine, because immunity from pigeon pox vaccine is not of long duration. c) In areas where pox is prevalent, fowl pox vaccine should be used for protection against infection from neighboring flocks.

In ovo *Vaccination.* Recent success with *in ovo* administration of FPV vaccines to 18-day-old chicken embryos has provided encouraging results (7, 8). With increasing use of *in ovo* vaccination, the cost of vaccination and stresses associated with handling the birds will be reduced significantly.

Recombinant Fowl Pox Virus Vaccines

Potential of FPV as a Polyvalent Vaccine

The pox viruses have some unique features (e.g., cytoplasmic site of multiplication, large genome, and unique viral enzymes and transcription system), which allow the expression of a foreign gene in a faithful manner. Thus, a large variety of genes encoding antigenic proteins of specific pathogens have been inserted into the genome of fowl pox virus. The genome of FPV is large enough to accommodate a significant amount of foreign DNA without a corresponding reduction in virus infectivity. The physical and biologic characteristics of FPV endow it with several advantages for use as an expression vector. First, fowl pox virus vaccines have been used in commercial poultry for more than 70 years. The vaccine virus causes a mild, localized self-limiting infection. In addition, FPV has a narrow host range affecting only avian species. The virus can be propagated in primary cultures of chicken embryo fibroblasts, chicken-embryo kidney cells, or skin cells, and also in permanent cell lines such as the Japanese quail cell line, QT35. Finally, because of its large size, genes from more than one pathogen can be inserted into its genome in order to create a polyvalent vaccine.

In order to develop a live recombinant FPV, it is important to insert stably the foreign gene(s) of interest from a poultry pathogen into the FPV genome, express the gene(s) optimally, and still maintain the infectivity of the virus. Thus, the generation of a FPV expression vector requires a) a suitable nonessential region in the FPV genome for insertion of foreign genetic material so that virus replication is not disrupted, b) a foreign gene that encodes for a protective antigen of a poultry pathogen, c) a strong pox virus promoter that will optimally regulate the expression of the inserted foreign gene(s), d) a donor plasmid that incorporates these three features, and e) a method for the selection and/or detection of recombinant progeny virus.

Nonessential Region

Several nonessential regions, including some in the terminal inverted repeats, have been identified in the FPV genome (1). One such commonly used insertion site for foreign sequences in the FPV genome is the TK gene (14, 89). Because TK activity is not required for avian pox virus multiplication, the encoding gene provides a convenient site for the insertion of a foreign gene(s). In addition, insertional inactivation of the TK gene reduces the virulence of the recombinant fowl pox virus (9) as compared to the unaltered parental virus. Other nonessential genes (e.g., those encoding photolyase, ATI protein, and a homologue of hemagglutinin) appear to be additional potential sites for the insertion of foreign genes.

Regulatory Sequences (Promoters)

Strong pox virus promoters are necessary for the expression of the inserted foreign genes. Pox virus promoters are relatively conserved and, thus, are recognized by heterologous pox viruses (14, 15, 90, 128). Initially, therefore, vaccinia virus promoters were used in lieu of FPV transcription regulatory elements in creating recombinant fowl pox viruses. Although homologous FPV promoters have since been identified (53, 54, 81) and a synthetic, early-late transcriptional regulatory element has been utilized, two vaccinia virus promoters, the early-late P7.5 and the late P11, have been used for the construction of recombinant avian pox viruses. In this regard, sequence analysis of the FPV genome (1) indicates the presence of 56 early, 3 intermediate, and 55 late putative promoters. Recently, several homologous FPV promoters, including a bidirectional one, have been evaluated (108, 110). Because some of these promoters appear to be as strong as the vaccinia virus ones, it is believed that their availability will assist in the development of a new generation of polyvalent FPV-vectored vaccines.

Donor Plasmid for Generation of Recombinant Virus

To create a recombinant virus, a donor plasmid that directs the insertion of foreign DNA into the FPV genome must be constructed. In such a plasmid, contiguous FPV DNA sequences are interrupted by a foreign gene(s) regulated by pox virus promoter(s). Following introduction of this plasmid into fowl pox virus-infected cells, *in vivo* recombination occurs in the cytoplasm between the homologous sequences of the replicating FPV genome and those that flank the foreign genes in the plasmid DNA. This interaction results in the insertion of the foreign transcriptional unit into the FPV genome.

Procedure for Selection of Recombinant Viruses

Because more than 99% of the progeny virus from a transfection is of parental type, a method for the selection and/or identification of recombinant virus is required. The recombinant viruses usually are identified and/or screened based on the expression of a marker gene that is inserted adjacent to the other foreign gene. The *Escherichia coli lac*Z gene has been widely used for this purpose, and the recombinant viruses are identified based on their ability to express ß-galactosidase (*lac*Z gene product) by the inclusion of the enzyme's histochemical substrates, X-gal or Bluo-gal, in agarose overlays of infected cells (81, 90). Plaques arising from infection by recombinant viruses appear blue, due to hydrolysis of these compounds, against a background of colorless plaques generated by nonrecombinant viruses. Alternatively, recombinants carrying the *E. coli* xanthine phosphoribosyl transferase gene as a marker can be selected due to their resistance to mycophenolic

acid (15). The green fluorescent protein (GFP) marker (41) can also be used for screening. In addition, recombinant viruses have been identified by plaque hybridization using a DNA probe specific for the inserted foreign gene.

Recombinant FPV Vaccines

Using the molecular techniques described previously, recombinant fowl pox or pigeon pox virus vaccines capable of producing proteins from the genes of several poultry pathogens have been created. Some of these antigens include the hemagglutinin of avian influenza virus, the fusion protein and the hemagglutinin-neuraminidase of Newcastle disease virus, the glycoprotein B of Marek's disease virus, the viral protein (VP) 2 of infectious bursal disease virus, and the nucleoprotein of infectious bronchitis virus. In most cases, foreign genes of avian pathogens inserted into the genome of avian pox virus are expressed, and the resultant proteins induce the generation of specific immunity against the respective pathogen.

Currently, a live FPV vectored vaccine "Avian Influenza-Fowl pox Vaccine" containing a cDNA copy of the H5 hemagglutinin gene of avian influenza is available commercially. It is recommended for immunization of chickens of one day of age or older birds by subcutaneous injection. Vaccinated birds remain immune to fowl pox for 10 weeks and immune to avian influenza subtype H5 for 20 weeks after the initial vaccination. It has been used rather extensively in Mexico.

A live FPV vectored vaccine "Newcastle Disease-Fowl pox Vaccine" for subcutaneous or wing-web stab immunization of one-day-old chickens is also available commercially. Similarly, a recombinant FPV vaccine expressing genes of infectious laryngstrocheitis virus has also become available.

Avian Pox Viruses as Expression Vectors for Genes from Mammalian Pathogens

The natural host range of avian pox viruses is limited to avian species. However, these viruses can initiate an abortive infection *in vitro* in cell lines of nonavian origin. Although infectious progeny virus is not produced, foreign antigens are synthesized authentically, processed, and presented on the cell surface. In this regard, expression of the rabies virus glycoprotein in recombinant FPV and canary pox viruses (118,119,120) provided a great impetus toward the use of avian pox viruses for the development of vaccines for both man and animals. For example, a canary pox virus-vectored vaccine expressing rabies virus glycoprotein G is currently available commercially for use in cats. Similarly, a recombinant canary pox virus vaccine expressing antigens of West Nile virus has been licensed for equines.

Treatment

No specific treatment exists for birds infected with avian pox viruses.

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Chapter 11

Reovirus Infections

Introduction

Richard C. Jones

Avian reoviruses are members of the Orthoreovirus genus in the family Reoviridae (5, 7). Members of the genus Orthoreovirus, family Reoviridae, share common physico-chemical properties and morphological characteristics (8). These include a double-stranded RNA (dsRNA) genome consisting of 10 segments packaged into a non-enveloped icosahedral double-capsid shell. The name "reovirus" derives from respiratory, enteric orphan, since they were first isolated from these sites in humans with initially, no apparent association with disease.

Orthoreoviruses can be classified based on distinct biological properties, most notably their host range and the unusual ability of certain members to induce cell-cell fusion, resulting in syncytia in cell culture. The Orthoreovirus genus consists of four species that separate into three distinct subgroups (15). The non-fusogenic mammalian orthoreovirus (MRV) species represents subgroup I. The fusogenic reoviruses separate into subgroup II, containing the avian reovirus (ARV)-type species and the Nelson Bay virus isolated from a flying fox, and subgroup III occupied by the baboon reovirus (BRV). Two syncytium-inducing isolates from snakes are defined as tentative species in the genus and form subgroup IV.

In addition to their fusogenic properties, avian reoviruses differ from mammalian reoviruses on the basis of their host pathogenicity and the lack of hemagglutinating ability (6). Avian reoviruses can be differentiated by antigenic configuration, pathotype, relative pathogenicity, growth in cell culture, sensitivity to trypsin, and host specificity (2, 3, 4, 5, 6, 10, 11, 12, 13, 14).

Although they are considered to be ubiquitous in commercial poultry and for the most part appear harmless, avian reoviruses have been isolated from a variety of tissues and organs in chickens affected by assorted disease conditions, including viral arthritis/tenosynovitis, stunting syndrome, respiratory disease, enteric disease, immunosuppression, and malabsorption syndrome (11, 12, 13, 14). They are frequently found in chickens that are clinically normal. The nature of the disease that occurs following reovirus infection is very much dependent upon host age, immune status, virus pathotype, and route of exposure. Interactions with other infectious agents have been documented (11, 13, 14) and may result in differences in both the nature and severity of reovirus-induced disease expression.

Reoviruses have also been associated with diseases in turkeys, ducks and geese and infection has been detected in several wild species of birds and these are dealt with in a separate section later in this chapter.

In young meat-type chickens, economic losses related to reovirus infections are frequently associated with increased mortality, viral arthritis/tenosynovitis (3, 11), and a general lack of performance including diminished weight gains, poor feed conversions, uneven growth rates, and reduced marketability of affected birds due to downgrading at slaughter (1). Breeder flocks that develop viral arthritis just prior to the onset of or during egg production may in addition to lameness be characterized by increased mortality, decreased egg production, suboptimal hatchability/fertility, and vertical transmission of virus to progeny. All of these can contribute to increased costs for poultry producers.

The best defined and most readily diagnosed reovirus-associated disease in chickens is viral arthritis (11), sometimes refered to as tenosynovitis (9). The disease has been recognized in virtually all major poultry-producing areas worldwide mainly as a disease of heavy meat-type chickens, but also in light breeds. Other disease conditions associated with reovirus infections can sometimes be demonstrated experimentally or are inferred by isolation from clinical accessions. However, these conditions are often difficult to recognize and conclusively diagnose in the commercial setting. Furthermore, experimental reproduction of the clinical condition is often difficult or impossible, so the reovirus-disease association is frequently tenuous. Because of the clear association between virus and disease and differences in disease expression, viral arthritis will be described separately in this chapter from other reovirus-associated diseases in poultry and other species.

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Viral Arthritis

Richard C. Jones

Introduction

Viral arthritis is an economically important disease of chickens that can be caused by different serotypes and pathotypes of avian reovirus (36, 46, 47, 55, 102, 105). The disease is considered to be most important in meat-type chickens but has from time to time been diagnosed in commercial layers (53, 116, 129). Although viral arthritis has been reported in turkeys (1, 2, 26, 83, 92, 119, 136, 149), experimental reproduction of joint lesions has not always been successful (1, 136).

The disease in chickens typically is controlled by vaccination with live attenuated and/or inactivated whole virus vaccines. Derivatives of the S1133 strain of reovirus are most commonly used as vaccines and have proven to be efficacious in most parts of the world. However, antigenically different reoviruses can break through vaccinal immunity. Autogenous vaccines can be used to provide protection against different serotypes (36, 109, 130). Turkeys and other avian species are not routinely vaccinated for viral arthritis.

Public Health Significance

No implications of public health significance have been reported.

History

In 1954, Fahey and Crawley (24) made what was later confirmed by Petek *et al.* (94) to be the initial isolation of avian reovirus from the respiratory tract of chickens with chronic respiratory disease. The Fahey-Crawley virus, when inoculated into susceptible chickens, produced a moderate respiratory disease, liver necrosis, and an inflammation of the tendons and synovial membranes.

Olson *et al.* in 1957 (91) described a naturally occurring synovitis in chickens from which they were able to isolate an agent insensitive to chlortetracycline and furazolidone and serologically unrelated to either *Mycoplasma gallisepticum* or *M. synoviae.* This agent, later named the "viral arthritis agent" by Olson and Kerr (87), eventually was identified as a reovirus by Walker *et al.* in 1972 (143). Dalton and Henry (15) used the term *tenosynovitis* (sometimes used synonymously with viral arthritis) to define the changes in the tendons and tendon sheaths associated with a condition they considered different from that caused by *M. synoviae.* This difference was substantiated by Olson and Solomon (89) when they reported tenosynovitis in commercially produced chickens that had been derived from *M. synoviae*-free broiler chickens. An isolate obtained from these birds had characteristics identical to those described for the "viral arthritis agent" and was shown to be antigenically similar to the Fahey-Crawley virus (90). Since the first reports of tenosynovitis in the United States and England, the disease has been described in many other countries. Several reviews document the incidence of reovirus-induced tenosynovitis (61, 99, 129).

Control of viral arthritis was greatly facilitated by recognition of the role of maternal antibodies in conferring protection to progeny (131, 135). Both viable and inactivated vaccines were subsequently developed to induce antibodies in breeder flocks for the protection of progeny from contact and transovarian transmission (131, 37, 138). The first commercially available live vaccines were developed by van der Heide *et al.* (133, 137) from the S1133 strain of avian reovirus. This same strain has been extensively used as an inactivated vaccine alone or in combination with other reovirus pathotypes.

Reoviruses that cause viral arthritis also have the potential to induce other pathological changes in chickens, particularly if introduced via the transovarian route or shortly after hatch (16, 48,

55, 78, 79, 101, 102). Disease conditions associated with some reoviruses that have arthrotropic characteristics include ruptured gastrocnemius tendons, pericarditis, myocarditis, hydropericardium, uneven growth, and mortality (37, 53, 60, 101, 102, 124, 129).

Incidence and Distribution

Reovirus infections are prevalent worldwide in chickens, turkeys, and other avian species. Viral arthritis is observed primarily in meat-type chickens but can be found in lighter breeds (53, 116) and turkeys (1, 5, 26, 83, 92, 119, 136, 149). It should be recognized, however, that reoviruses are commonly found in the digestive and respiratory tracts of clinically normal chickens and turkeys (58, 94, 1119, 149) and have been identified as a vaccine contaminant. It is estimated that greater than 80% of reoviruses isolated from chickens are non-pathogenic (130). This fact has implications in relation to diagnosis of reovirus infections.

Etiology

Virus Morphology and Structure

Reoviruses replicate in the cytoplasm, are nonenveloped with an icosahedral symmetry and a double-shelled capsid. Intact virus particles have a diameter of approximately 75 nm and a density in cesium chloride of 1.36–1.37 g/mL (29, 113, 114, 122). Their genomic dsRNA segments can be divided, on the basis of electrophoretic mobility, into three size classes: large (L1–L3), medium (M1–M3) and small (S1–S4). They code for at least 11 primary translation products separated into λ, μ and σ classes (142, 118).

ARV proteins have been less well characterized than their MRV counterparts. Most of the reported studies have concentrated on chicken strains of reovirus and concern σ proteins and S class-encoded genes. S1 encodes predicted polypeptides of 10, 17 and 35kDa, respectively. The p10 protein was recently described to be a non-structural protein responsible for the fusogenic property of the virus (118). The second has been suggested to play a role in pathogenicity (117). The 3′-terminal ORF encodes the minor outer capsid component expressed and located at the vertices of the spikes, σC displays cell-binding activity and inducing type-specific neutralizing antibodies (110). S2 encodes the major core protein, σA, suggested to play a role in resistance of ARV to interferon (73). S3 encodes the major outer capsid protein σB which reacts with an anti-reovirus polyclonal serum and is one of the group-specific neutralization antigens (110). Finally, S4 encodes the small non-structural protein, σNS, which may play an important role in the earliest stages of particle assembly (8, 159).

Gene M1 encodes a minor core protein μA, gene M2 a major capsid protein μB, and M3 μNS, a non-structural protein (85, 125). Proteins encoded by the L-class genes have received lesser attention.

Sequence analysis of selected S genes has led some authors to suggests that turkey and duck reoviruses should be ascribed to separate subgroups of avian reovirus from the chicken types (115), while the goose reovirus has similarities with the duck viruses (6).

Replication

The replication of avian reoviruses has been reviewed by Benavente and Martinez-Costas (9). Avian reoviruses enter cells by receptor-mediated endocytosis and acidification of virus-containing endosomes is necessary for the virus to uncoat and release transcriptionally active cores into the cytosol. They replicate within cytoplasmic inclusions of globular morphology, termed viral factories, which are not microtubule-associated, and which are formed by the nonstructural protein NS. This protein also mediates the association of some viral proteins (but not of others) with inclusions, suggesting that the recruitment of viral proteins into avian reovirus factories has specificity. Avian reovirus morphogenesis is a complex and temporally controlled process that takes place exclusively within viral factories of infected cells. Core assembly takes place within the first 30 min after the synthesis of their protein components, and fully formed cores are then coated by outer-capsid polypeptides over the next 30 min to generate mature infectious reovirions.

In the bird, replication of avian reoviruses has been described in various tissues but the chief targets appear to be the intestine (55), the tibiotarsal-tarsometatarsal (hock) joint (53) and the liver (62, 35).

Biophysical Factors

Reoviruses are heat resistant, being able to withstand 60°C for 8–10 hours, 56°C for 22–24 hours, 37°C for 15–16 weeks, 22°C for 48–51 weeks, 4°C for more than 3 years, −20°C for more than 4 years, and −63°C for more than 10 years (74). The titer of semipurified virus at 60°C is reduced, but not completely inactivated, in 5 hours. Heat treatment in the presence of magnesium chloride results in increased titers. The ability of avian reoviruses to survive on or in materials associated with the poultry house has been studied by Savage and Jones (112). They found that reoviruses survived for at least 10 days on feathers, wood shavings, egg shells and in feed but in drinking water virus was detectable for at least 10 weeks with little loss of infectivity.

Reoviruses are not sensitive to ether but are slightly sensitive to chloroform. They are resistant to pH 3; hydrogen peroxide when incubated for 1 hour at room temperature; 2% lysol; 3% formalin; and the DNA metabolic inhibitors actinomycin D, cytosine arabinoside, and 5-fluoro-2-deoxyuridine. They are inactivated by 70% ethanol, 0.5% organic iodine and a 5% solution of hydrogen peroxide (82). Sensitivity of avian reovirus to trypsin varies but is not related to antigenic configuration nor species of origin (47). The significance of trypsin sensitivity to relative pathogencity is unclear, but reoviruses sensitive to trypsin replicate poorly in the intestine following oral exposure and are not readily disseminated to other tissues sites (47, 81). Interestingly, S1133 derived vaccine viruses are inactived by trypsin treatment.

Avian reoviruses induce cell fusion, which differentiates them phylogenetically from most other animal reoviruses (18). Syncytia formation in cell culture increases the rate of reovirus

associated cytopathicity and virus released from infected cells but is not essential for either activity (19).

Despite two early reports to the contrary (19, 20), it is generally accepted that avian reoviruses, unlike their mammalian counterparts, do not demonstrate hemagglutination.

Strain Classification

Reoviruses can be classified using serologic procedures or grouped according to their relative pathogenicity for chickens. Kawamura and Tsubahara and Kawamura *et al.* (57, 58) identified five serotypes of reovirus from 77 isolates originally obtained from feces, cloacal swabs, and tracheas. Sahu and Olson (108) found four serotypes from intestines, respiratory tract, and synovial isolates. Wood *et al*. (147) calculated the relatedness of reoviruses originating from the United States, the United Kingdom, Germany, and Japan and found at least 11 serotypes, although there was considerable cross neutralization among heterologous types. Hieronymus *et al*. (37) grouped five reovirus isolates into three serotypes, and Robertson and Wilcox (98) assigned 10 Australian isolates into three groups with considerable cross-reactivity. It is apparent that reoviruses may frequently exist as antigenic subtypes, rather than distinct serotypes and reassortment can occur (98).

Rosenberger *et al.* (104) and Sterner *et al.* (124) inoculated specific-pathogen-free chickens by various routes with plaque-purified, antigenically similar viruses and demonstrated clear strain differences based on relative pathogenicity and virus persistence.

Laboratory Host Systems

Reoviruses grow readily in the embryonating chicken egg following inoculation via yolk sac or chorioallantoic membrane (CAM). The yolk sac is preferred for original isolation and generally results in embryo mortality 3–5 days after inoculation, with affected embryos exhibiting a purplish discoloration due to massive subcutaneous hemorrhage. Mortality in CAM-inoculated embryos usually occurs on day 7–8 PI; embryos are slightly dwarfed with occasional enlargement of the liver and spleen. Necrotic foci may occur in both the liver and spleen, particularly in embryos that survive longer than 7 days PI. Small, discrete, slightly raised white lesions may be found on the CAM. Histologically, areas of necrosis of the ectoderm with only moderate stimulation of the epithelial cells are seen. Mesoderm adjacent to the lesion is edematous and contains numerous inflammatory cells. Edema alone may be found. Embryo mortality is less consistent following inoculation via the chorioallantoic sac.

The virus grows in primary chicken cell cultures of embryo, lung, kidney, liver, macrophages, and testicle. Primary chicken kidney cells from 2–6-week-old chickens are satisfactory, but for plaques and isolation, primary embryo liver cells are preferred (30, 32). Chicken embryo fibroblasts are suitable for reovirus growth, but the virus often requires adaptation (7, 32, 54). Chicken-origin cell cultures infected with reoviruses are characterized by the formation of syncytia, which may occur as early as 24–48 hours, followed by degeneration, leaving holes in the monolayer and giant cells floating in the medium. Infected cells exhibit intracytoplasmic inclusions that may appear either eosinophilic or basophilic (99). Of many established cell lines tested, virus has been grown successfully, usually after adaptation, on Vero (108), BHK 21/13, 1TT, feline kidney (CRFK), Georgia bovine kidney (GBK), rabbit kidney (RK), porcine kidney (PK) (7), a Japanese quail cell line (QT35) derived from an induced fibrosarcoma (14), chicken lymphoblastoid cells (117), and subpopulations of chicken lymphocytes (80).

Pathogenicity

Although normally associated with arthritis, reoviruses have been strongly associated with other disease conditions in chickens as well, including growth retardation, pericarditis, myocarditis, hydropericardium, enteritis, hepatitis, bursal and thymic atrophy, osteoporosis and acute and chronic respiratory syndromes (24, 26, 47, 60, 83, 84, 101 124, 136). The pathogenicity of selected reovirus isolates was shown to be enhanced by coinfection with *Eimeria tenella* or *E. maxima* (106, 107). Exposure to infectious bursal disease virus or particular dietary regimes increased the severity of tenosynovitis resulting from infections with the WVU-2937 isolate (12, 13, 123). Reoviruses may also exacerbate disease conditions caused by other pathogens including chicken anemia virus (23, 76), *Escherichia coli,* and common respiratory viruses (97, 105). The increased susceptibility to other infectious agents following or concomitant with reovirus exposure may result from immune system compromise (97, 101, 105, 106, 107).

Pathogenesis and Epidemiology

Natural and Experimental Hosts

Although reoviruses have been found in many avian species, chickens are the only recognized natural or experimental hosts for reovirus-induced arthritis. The situation with turkeys is unclear. Reoviruses have been isolated from turkeys with arthritis (92), and Van der Heide *et al.* (136) found a turkey isolate to be pathogenic for chickens. The turkey isolate was neutralized by chicken reovirus S1133 antiserum. High mortality in turkey poults has also been associated with reovirus (119), although turkeys were shown by Al-Afaleq *et al.* (1) to be more resistant than chickens to reovirus-induced tenosynovitis. McFerran *et al.* (75) identified a reovirus in turkey feces that shared the group-specific antigen with chicken isolates but was not neutralized by available reference antiserum.

Reoviruses have been found in clinically affected ducks, pigeons, geese, American woodcock, and psittacine species, but a firm etiologic relationship was not always established (17, 75, 111). A disease in Muscovy ducks characterized by a general malaise, diarrhea, and stunted growth has been reported in several countries (25, 56, 71) and reproduced experimentally with isolated reoviruses (25, 71). Attempts to establish active infection in the canary, pigeon, guinea pig, rat, mouse, hamster, and rabbit failed; however, Phillips *et al.* (95) reported liver lesions in neonatal mice after oral and nasal infection, and Nersessian *et al.* (83) produced stunted growth and incoordination in suckling mice inoculated intracerebrally with several turkey isolates. Al-Afaleq *et al.* (3) showed that arthrotropic reovirus R2 caused

nervous signs and oiliness of the coat in mice, resembling the effect of mammalian reoviruses. Reoviruses in other avian species are discussed further in the second part of this chapter.

Age-Associated Resistance

Kerr and Olson (59) were the first to report an age-related resistance to reovirus-induced arthritis. The disease can be readily reproduced in 1-day-old chickens free of maternal antibody (48, 135), whereas older chickens are infected, but the disease is generally less severe, and the incubation period is longer. Similar results were reported by Rosenberger (104) with reoviruses isolated from birds with an apparent stunting syndrome and arthritis. Jones and Georgiou (48) suggested the age-associated susceptibility may be related to the inability of young birds to develop an effective immune response.

Transmission

Horizontal transmission of reovirus has been extensively documented (99, 129). There is considerable variation, however, among strains of virus in their ability to spread laterally. Although reovirus may be excreted from both the intestinal and respiratory tracts for at least 10 days postinoculation, virus generally appears to be shed from the intestine for longer periods, suggesting fecal contamination as a primary source of contact infection (53, 70). Roessler *et al.* (101) demonstrated that 1-day-old chickens are more susceptible to reovirus introduced via the respiratory route than orally. Virus may persist for long periods in the cecal tonsils and hock joints, particularly in birds infected at a young age (51, 72), implicating carrier birds as potential sources of infection for contacts.

Menendez *et al.* (78), Van der Heide and Kalbac (132) and Al-Mufarrej *et al.* (4) have clearly demonstrated that avian reoviruses can be vertically transmitted. Menendez *et al.* (78) showed that following oral, tracheal, and nasal inoculation of 15-month-old breeders, virus was present in chicks from eggs laid 17, 18, and 19 days postinfection. Reoviruses were also isolated from chicken embryo fibroblast cell cultures prepared from embryonated eggs derived from experimentally infected hens (102). Al-Mufarrej *et al.* (4) reported that virus-infected eggs were laid by virus-infected SPF hens between 5 and 17 days post infection and virus was isolated from the liver, intestine and hock joints of hatched chicks. Despite this, virus was never isolated from cloacal swabs taken from the hens. All reports suggest that the rate of egg transmission is low.

The possibility of avian reoviruses entering through broken skin in the foot and localizing in the hock joint was demonstrated using a trypsin-sensitive strain (2).

Incubation Period

The incubation period differs depending upon the virus pathotype, age of host, and route of exposure (99, 129). For inoculated 2-week-old chickens, the incubation period varied from 1 day (foot pad inoculation) to 11 days (intramuscular, intravenous, intrasinus inoculation). The incubation period following intratracheal inoculation and contact exposure was 9 and 13 days, respectively (89).

Often, infections are inapparent and demonstrable only by serology or virus isolations. Mature birds inoculated by oral and respiratory routes with the FDO isolate had virus in all organs tested at 4 days postinfection. The number of virus isolations was greatly reduced by 2 weeks, and no virus was present 20 days postinfection. There was frequent localization of virus in the flexor and extensor tendons of the pelvic limb, although gross lesions were not evident (79). Foot pad inoculation of 1-day-old chickens with an arthrotropic reovirus (R2) produced a more rapid progression of disease than either the oral, subcutaneous, or articular routes (43). When infected by the oral route, which appears to be a likely mode of naturally transmitted virus, the initial site of viral replication, which occurred within 2 to 12 hours postexposure, was the epithelium of the intestine and the bursa of Fabricius. This was followed by virus distribution in a wide range of tissues, including the hock joint, within 24–48 hours (55). Many reoviruses cause microscopic inflammatory changes in the digital flexor and metatarsal extensor tendons without development of gross lesions (88).

Chicks are most susceptible to reovirus infection when they are very young and the development of joint lesions is a slow process. When viral arthritis does result from naturally occurring infection, it is usually not seen in young birds before 4–7 weeks old but may be seen in much older chickens as well (129). In view of the increased resistance with age, the development of lesions at maturity is difficult to explain, unless persistent virus is reactivated by the stress of sexual activity. Morbidity can be as high as 100%, and mortality is generally less than 6%. The virus can persist in the tendons for at least 22 weeks (102).

Clinical Signs

In acute infections, lameness is present, and some chickens are stunted. With chronic infection, lameness is more pronounced, and in a small percentage of infected chickens, one or both hock joints are immobilized, preventing mobility. In a flock of 36,000 broilers, the infection, first diagnosed as infectious synovitis, appeared in 8 of 16 pens when the chicks were 3–4 weeks old. Approximately 550 birds died or were removed because of lameness by 7–8 weeks. Another 4,500 birds were stunted.

In another flock of approximately 15,000 broilers, no clinical signs of viral arthritis/tenosynovitis were observed, but approximately 5% of the birds had enlargement in the area of the gastrocnemius or digital flexor tendons when observed at slaughter. At 9 weeks, birds from this flock had an average weight of only 3.66 lb; feed conversion was 2.45; mortality totaled 5%; and the condemnation rate was 2.6%. Virus was isolated from two birds condemned for toxemia; of 80 serum samples obtained from this flock, 89% had reovirus antibodies detected in a precipitin test. This inapparent infection probably caused the poor performance of these broilers.

Similar observations have been made by other workers (229, 45). Rupture of the gastrocnemius tendon, especially in male roaster birds 12–16 weeks old, is often associated with reovirus infection (45, 54). A similar lesion has been seen in 5–8-week-old turkeys (92). The typical uneven gait in bilateral rupture of the tendon results from the inability of the bird to immobilize the

metatarsus. The latter is often accompanied by ruptured blood vessels.

Gross Lesions

Gross lesions in naturally infected chickens are observed as swellings of the gastrocnemius, digital flexor and metatarsal extensor tendons. The first lesion is evident by palpation just above the hock and may be readily observed when feathers are removed (Fig. 11.1). The affected joints usually feels warm. If the gastrocnemius tendon is ruptured, this can often be perceived as a greenish discoloration of the skin due to extravasation of blood. Removal of the skin will reveal the broken end of the tendon (51).



11.1. An 8-week-old broiler showing marked swelling of digital flexor and metatarsal extensor tendons. Diagnosis frequently can be made on the basis of the bilateral swelling of these tendons.



11.2. Marked edema of digital flexor tendon sheaths (left); normal (right).

Swellings of the foot pad and hock joint are less frequent. The hock usually contains a small amount of straw-colored or blood-tinted exudate; in a few cases, there is a considerable amount of purulent exudate resembling that seen with infectious synovitis. Early in the infection, there is marked edema of the tarsal and metatarsal tendon sheaths (Fig. 11.2). Petechial hemorrhages are frequent in the synovial membranes above the hock (Fig. 11.3A).

Inflammation of tendon areas progresses to a chronic-type lesion characterized by hardening and fusion of tendon sheaths. Small pitted erosions develop in the articular cartilage of the distal tibiotarsus. These erosions enlarge, coalesce, and extend into underlying bone (Fig. 11.3B,C). An overgrowth of fibrocartilaginous pannus develops on the articular surface. Condyles and epicondyles are frequently involved (60). In inoculated chickens, the diaphysis of the proximal metatarsal of the affected limb is enlarged.

Histopathology

Histologic changes have been described by Kerr and Olson (59). In general, they are the same for naturally occurring and experi-



11.3. Viral arthritis lesions in distal posterior tibia of inoculated chickens. A. Normal.



11.3. (*continued*) B. Cartilage erosions and hemorrhages of synovial membrane 35 days postinoculation. C. Erosions of cartilage and marked thickening of synovial membrane 212 days postinoculation.

mental infections. During the acute phase (7–15 days following foot pad inoculation), edema, coagulation necrosis, heterophil accumulation, and perivascular infiltration are seen. There also are hypertrophy and hyperplasia of synovial cells, infiltration of lymphocytes and macrophages, and a proliferation of reticular cells. These latter lesions cause parietal and visceral layers of the tendon sheaths to become markedly thickened. The synovial cavity is filled with heterophils, macrophages, and sloughed synovial cells. Periostitis characterized by increased osteoclasts develops. During the chronic phase (starting by 15 days postinfection), the synovial membrane develops villous processes, and lymphoid nodules are seen. After 30 days, inflammatory changes become more chronic. An increase in the amount of fibrous connective tissue occurs, and a pronounced infiltration or proliferation of reticular cells, lymphocytes, macrophages, and plasma cells also can be seen.

The same general inflammatory changes develop in the tarsometatarsal and hock joint areas. Development of sesamoid bones in the tendon of the affected limb is inhibited. Some tendons are replaced completely by irregular granulation tissue, and large villi form on the synovial membrane.

At 54 days postinfection, orally infected birds showed chronic fibrosis of tendon sheaths, with fibrous tissues invading tendons and resulting in ankylosis and immobility (134).

Linear growth of cartilage cells in the proximal tarsometatarsal bone becomes narrow and irregular. Erosions on the hock joint cartilage are accompanied by a granulation pannus. Osteoblasts become active and lay down a thickened layer of bone beneath the erosion. Osteoblastic activity is present on the condyles, epicondyles, and accessory tibia, producing osteoneogenesis and subsequent exostosis (60). Ultrastructurally, the gastrocnemius tendon and sheath in broilers infected with reovirus at 1 day of age by the oral route were characterized by degenerative changes in fibroblasts including cytoplasmic vacuolization, membrane disruption, loss of ribosomes from the endoplasmic reticulum, and generalized mitochondrial and cellular disruption (39).

Lesions found in the heart have been described in detail (60, 89). An infiltration of heterophils between myocardial fibers is a constant finding. In some cases, it is accompanied by proliferating mononuclear cells, probably reticular cells. However, it is not clear whether this is pathognomonic of reovirus infection. The pathogenicity of avian reoviruses for day-old chicks revealed the arthrogenic potential for many strains and marked hepatic necrosis (32). Erythrocyte, hematocrit, and total leukocyte determinations are generally within the normal range, although there may be a rise in the heterophil percentage and a decrease in the lymphocyte percentage.

Immunity

In the relatively few reports on the mechanisms of immunity to avian reoviruses, both joint and enteric effects have been studied.

Humoral Antibodies

Avian reoviruses possess a group-specific antigen discernable with gel diffusion techniques (146) and a serotype-specific antigen demonstrable with neutralizing antibody in plaque-reduction or chicken embryo assays (99, 129). Neutralizing antibodies can be detected 7–10 days following infection, and precipitating antibodies at approximately 2 weeks. Neutralizing antibody appears to persist longer than precipitating antibody, but this may be a reflection of assay sensitivity. The importance of antibody in establishing protection is not well understood, because birds may become persistently infected in the presence of high levels of circulating antibody (52). It is clear however, that maternal antibody can afford a degree of protection to 1-day-old chickens against naturally occurring and experimental challenges (133, 135). Relative protection afforded by antibody appears to be related to serotype homogeneity, virus virulence, host age, and antibody titer (96, 104, 126, 135, 144).

Local Antibodies

Induction of intestinal virus-specific IgA, which may be important in limiting the pathogenic potential and dissemination of reovirus, is affected by route of exposure, age, and sensitivity to trypsin (81). Chickens infected at one day of age or with trypsin-sensitive reovirus by the oral route do not have a detectable intestinal IgA response.

Cell-Mediated Immunity

In an early report on cell-mediated immunity, Kibenge *et al.* (62) using immunosuppression by bursectomy and/or thymectomy of chicks showed that recovery from reovirus infection involves both B- and T- cell systems but that protection is predominantly B-cell mediated. In birds treated with both, virus infection appeared more persistent. Hill *et al.* (38) reported that the suppression of T-cell-mediated immunity by cyclosporin A resulted in increased mortality in reovirus-infected birds, but the relative severity of tendon lesions was unaffected.

In a study of the enteric activity of several strains of reovirus, Songserm *et al.* (121) showed that CD8+ T-cells may play a major role in the pathogenesis and/or reovirus clearance in the small intestine. van Loon *et al.* (139) showed that in B-cell immunosuppressed chicks, challenge virus is controlled in the absence of actively produced antibodies, and is independent of B lymphocytes. This suggests that cellular immunity is sufficient for protection of broilers with maternal antibodies against reovirus infection following early age vaccination with live reoviral vaccine.

Pertile *et al.* (93) used monoclonal antibodies specific for B and T lymphocytes to study the development of reovirus arthritis. T-cells and plasma cells were the predominant inflammatory cells in the synovium. In the acute phase, T-cell, mostly $CD8^+$ were present in low numbers. Most activity was in the subacute phase with increased numbers of $CD4^+$ and $CD8^+$. Aggregates of cells, IgM-positive B-cells and plasma cells were also present. The chronic stage was characterized by large numbers of predominantly $CD4^+$ cells. These changes were considered similar to those seen in rheumatoid arthritis in humans. These results indicated that a potential consequence of macrophage priming following virus infection is the protection of cells against virus-induced replication and cytopathic effects, and this protection may be mediated by the cytostatic effects of nitrous oxide on the host cell. Further studies on spleen cells from reovirus infected birds indicated that reovirus infection in chickens does not compromise the functional capabilities of T-cells but induces suppressor macrophages that inhibit T-cell functions (Pertile *et al.* (93). Supporting evidence for the rheumatoid arthritis comparison was provided by the detection of anti-nuclear and anti-collagen antibodies in some reovirus-infected birds (44).

Interferon production by avian reoviruses has been demonstrated *in vitro* and *in vivo*. The S1133 attenuated strain induced interferon in chick embryo cell cultures, and *in vivo* interferon was detected in the lungs but not in other tissues (21, 22, 145). A more pathogenic reovirus elicited the production of interferon detectable in serum samples (21, 22).

Diagnosis

A presumptive diagnosis of viral arthritis may be made on the basis of signs and lesions. However, the signs of viral arthritis are not pathognomonic and some joint lesions may resemble those caused by *Mycoplasma synoviae,* staphylococci or other bacteria. Involvement of primarily the metatarsal extensor and digital flexor tendons (see Fig. 11.2), and heterophil infiltration in the heart, assist in differentiating reovirus infection from similar disease caused by these other agents. However, demonstration of virus in clinical material is required to confirm the cause. Hitherto, this has meant virus isolation, but more recently, more rapid molecular methods and especially the polymerase chain reaction have been developed.

Detection of Virus

Virus Isolation

Avian reoviruses are normally among the easiest of viruses to grow, so it is often tempting to consider them as the cause of a clinical condition, when they may actually play little or no part in it.

It is recommended that for isolation from the joints, tissue samples are more likely to yield virus than swabs (49). Pieces of

hypotarsal sesamoid bone with the tendons that pass through it, synovial membrane and articular cartilage are the tissues of choice. Joint material should be taken from apparently normal birds in addition to those that are affected, since the number of clinically affected birds in a flock at any one time may be relatively small. Also, virus isolation from joints in advanced stages of degeneration may be impossible. It should be remembered that since avian reoviruses are ubiquitous and for the most part, harmless, isolation of virus from the intestine is likely to be meaningless in interpreting the cause of joint lesions. Specimens should be sent to the laboratory in transport medium even though the virus is relatively resistant. If a delay occurs, specimens can be stored at 4°C temporarily or for longer periods at –20°C or below.

Virus isolation is best achieved from hock joint tissue using chick embryos after 5–7 days of incubation inoculated via the yolk sac, or chicken embryo liver cells (32, 99, 129). Reoviruses typically kill the embryos within 5–6 days and in cell cultures, syncytium formation on the cell sheet is the typical effect. More than one passage in eggs or cell cultures may be needed.

Reoviruses can be readily differentiated from other viruses by their typical physicochemical characteristics and the presence of a group-specific antigen demonstrable with the agar gel precipitin test. For preparation of the antigen, 9–11-day-old embryonating chicken eggs are inoculated by the CAM route, and CAMs are harvested from dead or affected embryos within 7 days postinoculation. The CAMs then are homogenized and used as antigen (90). The precipitin test can be used to identify isolates as reovirus if known positive antiserum is available, or it can be used as an indication of antibody status in affected flocks. Isolated virus can also be identified by its typical 70–80nm diameter double-shelled morphology under the electron microscope, or by immunofluorescence staining of infected cell sheets.

Molecular Methods

Molecular approaches to identification of avian reoviruses in infected tissues have been described by several authors and are now being used in some diagnostic laboratories. These include dot-blot hybridization (66, 151) and, more commonly, reverse transcriptase-polymerase chain reaction (RT-PCR) (63, 67). Liu *et al.* (69) contended that RT-PCR followed by restriction enzyme fragment length polymorphism (RFLP) provides a simple and rapid approach for characterization of ARV isolates. Also, it is possible to determine whether a new variant strain has been introduced into a flock or a given virus strain has spread from one flock to another. Caterina *et al.* (11) developed a multiplex PCR for reovirus, adenovirus, infectious bursal disease virus and chicken anaemia virus. RT-PCRs have also been found useful for screening poultry vaccines for extraneous avian reoviruses in the quality control of biologicals used in veterinary medicine (10).

Undoubtedly the molecular methods and especially RT-PCR are rapid and combined with RFLP, enable molecular epidemiology to be investigated. However, for the routine examination of field material, it is important that their sensitivity is compared critically with that of virus isolation, which can still be considered as the "gold standard" for diagnosis of avian reoviruses. It should also be remembered that RT-PCR diagnosis does not provide the live virus, so isolation needs to be done if a new isolate is to be investigated further.

Location of Virus in Tissues

Lesion-associated reovirus proteins or nucleic acid can be detected in formalin-fixed tissue using immunoperoxidase procedures (127). Demonstration of reovirus antigens in cryostat sections of snap-frozen tendon sheaths and other tissues by fluorescent antibody staining (99) is a rapid alternative method of diagnosis but is probably useful only in the early stages of infection (66). Liu and Giambrone (66) described the use of *in situ* hybridization for tracing avian reovirus strain S1133 in tissues of infected chickens. They were able to detect virus in wax-embedded sections of liver, pancreas, heart and tendon, thus confirming the target sights for these viruses. Both immunmostaining and *in situ* hybridization are of course well suited to pathogenesis studies, but could be used to determine the tissue tropism of a new isolate.

In spite of extensive molecular studies, there are no recognized markers for pathogenicity of avian reoviruses, so experimental infections are required. For a reovirus obtained from an affected joint, its arthrotropic potential can be confirmed by inoculation into the foot pad of susceptible 1-day-old chicks. If pathogenic, the virus will induce a pronounced inflammation of the foot pad within 72 hours postinoculation.

Serology

Reovirus group-specific antibodies can be detected readily with the agar gel precipitin test (57, 97) or indirect fluorescent antibody (IFA) assay (31). The IFA test is more sensitive and, therefore, better suited for quantitative evaluations. Virus neutralization, based on plaque reduction in chicken kidney or chicken embryo liver cell cultures and several cell lines, has been routinely used for determining serotype differences with rabbit or chicken antiserum and monoclonal antibodies (58, 64, 126, 144, 147). Although several serotypes have been described, considerable homogeneity exists among reovirus isolates, with many being classified as antigenic subtypes rather than distinct serotypes. *In vitro* measurements of reovirus antibody specificity may not always correlate with protection against homologous and heterologous challenge of birds with maternally derived antibody (148), and the type specificity of neutralizing antibody is less for chickens immunized with inactivated reovirus than for chickens immunized following infection (77). None of these tests is suitable for large-scale screening.

For commmercial purposes, the above tests have been superseded by enzyme-linked immunosorbent assay (ELISA) as the serological method of choice, allowing automation and rapid testing of many samples. Slaght *et al.* (120) were the first to describe an ELISA for detecting avian reovirus antibody. The S1133 strain was used as antigen and found to react with antibodies to the Reo-25 and WVU-2937 isolates, although homologous antibody gave the highest titer. Islam and Jones (42) found a significant correlation between ELISA and virus neutralizing antibodies. The ELISA systems now available from commercial sources are used widely for assessing reovirus antibody levels on

a flock basis (128). Recently reported refinements have included the use of bacterially expressed σC and σB proteins (68) as ELISA antigens to improve assessment of flock immune status and monoclonal antibody-based competitive ELISA (65).

Since reovirus infection is widespread among commercial flocks, the diagnostic value of serological profiling is often difficult to interpret, although it can be an indicator of immunity following vaccination.

Prevention and Control

Biosecurity

Avian reovirus infections (but not necessarily disease) are ubiquitous. The virus can be transmitted both vertically and horizontally and, because of its resistance to inactivation away from the host, may be frequently carried by mechanical means. In view of these properties, maintaining freedom from infection in modern intensively housed chicken flocks appears to be virtually impossible. Following the removal of an infected flock from the premises, thorough cleaning and disinfection of a poultry house can prevent infection with pathogenic virus in subsequent groups. Because of the relative stability of the avian reovirus group, commercially available disinfectants should be validated for efficacy before use. Lye and 0.5% organic iodine solutions are considered to be effective inactivating agents. Though the main approach to control of reovirus infections in chickens is vaccination, good management and biosecurity procedures which minimize reovirus infections, especially in young chickens, are essential.

Vaccination

Chickens are most susceptible to pathogenic reoviruses at 1 day of age and then develop an age-associated resistance beginning as early as two weeks. Because of this enhanced period of susceptibility, vaccines and vaccination programs have evolved that are directed at providing protection at 1 day of age. Active immunization can be achieved by vaccination with viable attenuated reovirus that is usually applied by the subcutaneous route (137), although immunization by coarse-spray application of vaccine has also been used (28). Protection from subsequent challenge can be demonstrated, but the S1133-derived reovirus vaccines may interfere with Marek's disease vaccination if administered simultaneously (103, 86). The interference is most pronounced with herpes virus of turkeys (HVT) derived Marek's disease vaccines (97, 103). A reovirus vaccine derived from a naturally apathogenic strain of reovirus (2177) isolated in the United States (101, 102, 124) may be more suitable for simultaneous day-of-age administration with Marek's disease vaccine than are several S1133 derivatives (138). The vaccine should be used with caution if Marek's disease vaccine titers are low and/or Marek's disease virus challenge is significant. Reovirus vaccination of breeding stock can be done with viable or inactivated vaccines or combinations of both. The inactivated vaccines are more efficacious if preceded by vaccination with live vaccine (130, 148).

If a live vaccine is used, it should be administered prior to the onset of egg production to prevent transovarian transmission of the vaccine virus (27). The advantages of this type of immunization program include immediate protection of 1-day-old progeny provided by maternal antibody and a limitation of the potential for vertical transmission that has been shown to be economically significant (16). Vaccination of breeders is an efficacious method of controlling viral arthritis and other pathogenic reoviruses, but it should be recognized that protection is assured against homologous serotypes only (36, 96, 109). When the field virus is clearly different from that included in commercially available vaccines, an autogenous vaccine may prove effective (36, 109).

In ovo vaccination has been investigated. Guo *et al.* (33, 34) reported that the reovirus vaccine alone should not be given to chicks without maternal antibodies but this immunosuppression could be overcome if the vaccine was given with antibody complex.

Among the novel approaches to reovirus vaccination, Wu *et al.* (151) expressed σC protein in yeast (*Schizosaccharomyces pombe*). Projection against S133 challenge was slightly superior to that of a commercial vaccine. The results supported the feasibility of plant-derived vaccines for immunization programmes. Huang *et al.* (40) in similar work, expressed the σC protein in alfalfa and possibly paved the way for an edible vaccine.

The accepted method of assessing efficacy of reovirus vaccines has been to vaccinate birds and 3–4 weeks later, challenge them with virulent virus via footpad inoculation. Results are based on interpretation of the footpad swellings. This causes suffering in the challenged birds and results can sometimes be difficult to interpret. van Loon *et al.* (141) have described an alternative challenge model which involves isolation of the challenge virus from selected tissue of vaccinated or control birds and, using monoclonal antibodies, discriminating challenge virus from vaccine virus.

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Other Reovirus Infections

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Introduction

Reoviruses are recognized as a cause of tenosynovitis/viral arthritis in chickens (see preceding section, "Viral Arthritis"), but they have also been isolated from several other disease conditions in chickens and turkeys. From time to time, they have been recovered from a variety of other healthy or diseased avian species. In many cases, attempts to demonstrate that the reoviruses were the cause of the condition have been unsuccessful, or, especially in the case of wild species, experimental work has not been possible. Thus, their association with disease has not always been established. On other occasions, serological surveys have been conducted, which have shown the presence of antibodies to at least the avian group antigens in other avian species. In these cases, the role of reovirus infection is completely unknown.

Some strains isolated from birds other than chickens and turkeys, however, have been shown capable of causing patholog-

ical changes (mainly in the hock joints) in chickens, suggesting the possibility of cross-species transmission (33). The role of other avian species as carriers and reservoirs of infection for domestic poultry has never been established.

The Viruses

In almost all cases in which reoviruses have been isolated, cultivation has been achieved using methods described for the viral arthritis strains—namely, fertile chicken eggs inoculated via the yolk sac, or chick embryo fibroblasts, liver or kidney cells, or chicken kidney cells. Isolates usually have been identified by cultural characteristics and typical reovirus morphology under the electron microscope. Few comparisons have been made of reoviruses from viral arthritis and those from other conditions of poultry or other avian species.

Rekik *et al.* (60) examined reoviruses isolated from 9 flocks of broiler chickens in Quebec. Serum neutralization tests showed the presence of types antigenically different from the vaccine (S1133) strain. They asserted that some reoviruses isolated from conditions other than viral arthritis could be antigenically different. Heffels-Redmann *et al.* (28) examined two reoviruses isolated from Muscovy ducks. Although the basic electrophoretic mobility patterns of immunoprecipitated polypeptides closely resembled those of chicken strains, considerable strain-specific variation was seen at the protein level. Based on cross-neutralization tests, the two duck strains were grouped in one serotype, with no cross-reactivity with the chicken serotype S1133.

Lozano *et al.* (45), using polyacrylamide gel electrophoresis, compared the genomic profiles of a total of 70 avian reovirus isolates, comprising 60 from turkeys, 8 from chickens (including strain S1133) and one each from a canary and a cockatiel. Greater heterogeneity of the migration pattern was seen among the turkey reoviruses as compared with the 8 chicken viruses, particularly in the S (small) genome fragments. A characteristic migration pattern according to species could not be determined because of the high polymorphism existing in the mobility patterns from chickens and turkeys. However, the canary and cockatiel viruses had strikingly similar migration patterns, which were different from the chicken and turkey viruses. The authors indicate the difficulty of assessing the significance of these differences, because these two viruses were processed separately, and virus isolation time was different.

Further detailed comparisons are needed between reovirus strains from different avian species.

Diseases in Chickens

Reoviruses have been isolated from a wide range of disease conditions in commercial chickens other than tenosynovitis. They include respiratory disease, enteric disease, inclusion body hepatitis, hydropericardium, hepatitis in young chicks, generalized disease, blue wing disease, and the runting/malabsorption syndrome. At the same time, they can be isolated easily from the intestines of apparently healthy chickens. In addition to differences in tissue tropism between strains, a range of virulence exists, from high to virtually harmless. Several reports describe varying degrees of reduced weight gain due to reovirus infection, presumably indicating varying effects on function of the gastrointestinal tract.

A study by Robertson *et al.* (62) investigated the presence of reoviruses in healthy commercial chickens and in other flocks affected with the runting syndrome or tenosynovitis. The viruses could be isolated from almost all fecal samples from healthy flocks of 3 weeks of age or older, from several tissues of chicks aged 2 weeks or more with the runting syndrome, and from older birds with tenosynovitis. In addition, all broiler breeder flocks examined had antibodies to avian reovirus. The finding of widespread reovirus infection, apparently in the absence of disease, strongly suggests that isolation of reovirus from tissue specimens does not necessarily imply that they are causing disease.

Kant *et al.* (35) sequenced part of the S1 segment of the σC protein of avian reoviruses from chickens isolated from a variety of disease conditions in Germany and the Netherlands between 1998 and 2000. They were unable to correlate σC sequences with different disease conditions and did not establish temporal or geographic differences either. Thus the underlying markers that identify pathotype or tissue tropism are still to be investigated.

Reports of reoviruses being associated with conditions in chickens other than joint disease include the following.

Respiratory Disease

The so-called Fahey-Crawley virus (18), whose identity was later confirmed as the first avian reovirus (59), caused a mild respiratory disease of baby chicks (73), but older chicks were resistant. Another respiratory isolate (UGA) was unable to cause respiratory disease alone, but in combination with a strain of *Mycoplasma gallisepticum* of low pathogenicity, respiratory signs and lesions were observed (69). However, reoviruses generally are not regarded as primary agents of respiratory disease in poultry.

Enteric Disease and Systemic Infections

Several descriptions exist of reovirus-associated enteric disease. An agent characterized as a reovirus was isolated from young chicks suffering from ulcerative enteritis by Krauss and Ueberschar (40), but it was not confirmed that this virus was the cause of the disease. Further early reports described enteric disease (14) and cloacal pasting and mortality (16) in young chicks.

A commercial farm with a history of poor feed conversion and chronic feed-passage problems was investigated (3). Abnormal tissue pathology was seen in broilers from 9 days of age. Avian adenoviruses and reoviruses were isolated, and although SPF chicks were inoculated with isolated reoviruses, their relationship to the initial problem was inconclusive.

Adenoviruses and reoviruses isolated from commercial broiler chickens were tested for gastrointestinal pathogenicity in day-old chicks by Lenz *et al.* (43). Chickens in inoculated groups developed wet unformed fecal droppings, but although adenoviruses caused marked gizzard erosions, necrotizing pancreatitis, and proventriculitis, reovirus effects were mild by comparison, including hyperplasia of lymphoid aggregates and mild gizzard erosions.

Some reports highlight the synergistic effect of reovirus in dual infection with other pathogens. For example, reovirus and *Cryptosporidium baileyi* produced a systemic infection (26), and Ruff and Rosenberger (66) showed that reoviruses can potentiate coccidial infection, although the outcome depends on the reovirus strain used.

Other reports record more generalized infections, with several organs affected. Four outbreaks of disease in broiler chickens in Victoria, Australia, were examined by Bagust and Westbury (4). Affected flocks range from 4–38 days. Sudden deaths and starvation were variously associated with hepatitis, ascites, hydropericardium, pale kidneys, and depleted bursas. Reoviruses were isolated consistently from the tissues of the affected birds. Inoculation of these viruses into day-old SPF chicks intraperitoneally or orally induced sporadic deaths but no clinical syndromes. It was speculated that other factors may interact with the reoviruses to induce these problems.

Blue wing disease is a condition affecting broilers characterized by mortalities of 10%, subcutaneous and intramuscular hemorrhages, and atrophy of the thymus, spleen, and bursa. Engstrom *et al.* (17) showed that it was caused by a synergistic effect between chicken anemia virus and a reovirus. McNeilly *et al.* (48) also showed a synergistic effect between these two agents, so that dually infected animals had significantly lower weight gain and more severe damage in several tissues than chicks inoculated with either alone. However, the severity of effects depended on the strain of reovirus.

Avian reoviruses have also been shown to enhance the pathogenicity of other infectious agents of chickens such as *Escherichia coli* (64) and infectious bursal disease virus (51).

Inclusion Body Hepatitis; Hepatitis in Young Chicks

The liver of the chicken is considered to be one of the target organs for reovirus infection. It has been shown experimentally that reoviruses can cause hepatitis immunosuppressed chicks (38). McFerran *et al.* (47) isolated reoviruses and adenoviruses from outbreaks of inclusion body hepatitis. However, it is now recognized that adenoviruses rather than reoviruses play an important role in the pathogenesis of this disease.

Mortality in broiler chicks up to 10 days of age has been reported in Poland and attributed to reoviruses (Z. Minta, personal communication). Van Loon *et al.* (75) characterized these viruses and called them "enteric reovirus strains" (ERS). The pathogenicity, dissemination, induction of malabsorption syndrome, unusual reaction pattern with different monoclonal antibodies to more common reoviruses, and serotype properties were reported. Screening of reoviruses in the field showed that these reovirus strains were also present in other countries and were usually isolated from birds with MAS. The authors proposed that the so-called ERS are associated with MAS. Whether these viruses can be differentiated from other chicken strains by molecular methods remains to be seen.

Hydropericardium

Bains *et al.* (5) described serious mortalities (10–18%) in broiler chicks in Queensland, which occurred in birds less than 14 days old. At necropsy, hydropericardium, with in some cases up to 3 ml fluid, was a consistent feature together with small spleens. Reoviruses were isolated from the hearts of these birds in fertile eggs or cell cultures, but there was no attempt to show that reoviruses were the cause of the condition. However, Jones (32) described a similar investigation but showed that intravenous inoculation of the isolated reovirus induced hydropericardium in experimentally infected chicks. The pathogenesis of this condition in relation to reoviruses has never been studied.

The Runting-Stunting/Brittle Bone Disease/Malabsorption Syndrome in Broilers

A disease syndrome with several names that first appeared in broilers in the late 1970s and chiefly is characterized by lowered body weights and variously described as the runting-stunting, pale bird, malabsorption syndrome (MAS), brittle bone, helicopter wing syndrome has been linked with several possible causative agents, including reoviruses (12, 22, 55, 58, 63, 75, 76). However, several studies have suggested that reoviruses probably play a secondary role in these conditions rather than a primary one. Montgomery *et al.* (50) attempted to reproduce the syndrome using various infectious agents isolated from affected Mississippi broilers. These included an infectious bronchitis virus (IBV) isolate and a reovirus. Although IBV with the reovirus caused weight depression, it was concluded that none of the isolated agents was the ultimate cause.

The enteropathogencity of avian reoviruses isolated from chickens with malabsorption syndrome from the Netherlands and Germany was studied by Songserm *et al.* (71). Despite replicating in the intestinal epithelium and causing small intestine lesions including denuding of the villi, none of the viruses caused weight gain depression. The authors concluded that reovirus alone cannot induce intestinal lesions as found in malabsorption syndrome. However, this group (Songserm *et al.*, 2002) had previously shown that a combination of enteropathogenic reovirus with other agents or substances present in an intestinal homogenate from affected and healthy chickens can induce MAS in broilers. *E. coli* was not essential for induction of weight gain depression but played a part in development of intestinal lesions, which alone do not always result in weight gain depression.

Van Loon *et al.* (75) characterized the Polish reoviruses associated with mortalities in baby chicks and hepatitis. They called them "enteric reovirus strains" (ERS). The pathogenicity, dissemination in the tissues, induction of malabsorption syndrome and in particular, their unusual reaction pattern with different monoclonal antibodies to other reoviruses were reported. These viruses were found in other countries and usually isolated from birds with MAS. The authors propose that ERS reoviruses are associated with MAS.Whether these reoviruses can be differentiated from other chicken strains by molecular methods remains to be seen.

So, although several reports indicate that isolated reoviruses are sometimes capable of causing varying degrees of enteritis (65, 75), or simply reduced weight gain (65, 74), the consensus view appears to be that the most important pathogen is a small virus that can be seen in the enterocytes but which has escaped cultivation (50).

Nonetheless, some commercial reovirus vaccines are produced, which are claimed to have beneficial effects against the stunting or malabsorption syndrome. The claims may indeed have some justification, but the vaccines are unlikely to protect against the primary causes.

Diseases in Turkeys

Tenosynovitis

Reoviruses have been isolated from tenosynovitis in turkeys (44, 56), but the relationship of the viruses with this disease is unclear. Al-Afaleq and Jones (1) examined three chicken and three turkey reoviruses each isolated from hock joints. All viruses induced microscopic tenosynovitis lesions in chicks, but none produced them in turkey poults. Even when reovirus was given together with *Mycoplasma synoviae*, only minimal joint lesions were induced in experimentally infected turkeys (2).

Enteric Disease

Reoviruses have been isolated from the intestines of normal turkeys and turkeys with enteric disorders (13, 20, 53, 54, 68). When such strains have been tested *in vivo*, effects have been variable: Some have been found to be pathogenic, and others nonpathogenic or of low pathogenicity. Dees *et al.* (13) compared different isolates and found that although strain BC-7 was nonpathogenic, BC-3 induced enteritis, involving destruction of the intestinal villi. Goodwin *et al.* (21), using a brilliant red powder in the diet, found that gastrointestinal transit time in reovirus-infected turkeys was significantly longer than in normal turkeys.

In the USA a condition in young turkeys called poult enteritis and mortality syndrome (PEMS) caused major losses to the turkey industry especially in the 1990s. Little is known about its incidence outside the USA but is has recently been reported in the United Kingdom (10). PEMS was originally called poult enteritis complex (7), and its main features include stunting and poor feed utilization that result from enteritis. In the more severe forms, runting, immune dysfunction, up to 100% morbidity and mortality are reported. Gross microscopic lesions of enteritis are present in all forms but tend to be non-specific. The etiology of the disease is not completely understood but it appears to be multifactorial. While the agents thought to be most important are enteropathogenic *E. coli*, turkey coronaviruses (27) and turkey astroviruses (39) and reoviruses (29) have also been isolated. Heggen-Peay *et al.* (29) showed that while experimentally, PEMS reovirus isolate ARV-CU98 did not induce fulminating PEMS in turkey poults, it was able to cause some of the typical clinical signs including intestinal alterations and significantly lowered liver and bursa weights. The authors considered that it could also contribute indirectly to the syndrome by increasing susceptibility to opportunisitic pathogens that facilitate clinical PEMS. Sequence analysis of reovirus NC98 isolated from PEMS and other turkey strains (36) has shown that they shares limited sequence analysis with reoviruses of chicken or duck origin and should be considered to constitute a separate virus species within subgroup II of the Orthoreovirus genus.

The implied involvement of the three types of virus in PEMS led to the development of a multiplex real-time RT-PCR by Spackman et al., (72) for turkey coronavirus astrovirus and reovirus which is claimed to be as sensitive as isolation for each of the viruses.

Reoviruses in Ducks and Geese

Several early reports describe the isolation of reoviruses from different species of ducks, including mallards (46), healthy Pekin ducks (33), and diseased ornamental ducks (23). All shared a common group antigen with chicken reoviruses, but their relationship with disease in ducks was not determined. However, the strains from Pekin ducks (33) were able to cause microscopic lesions of tenosynovitis in specific-pathogen-free chicks. Heffels Redmann *et al.* (28) considered that the two duck strains they examined were antigenically distinct from standard chicken strains.

Muscovy duck reovirus is the cause of a disease first described in South Africa and then France (1972) and typically affects young ducklings 2–4 weeks of age, causing diarrhea and difficulty in movement, high morbidity and mortality of 10% or higher. Malkinson *et al.* (49) isolated a reovirus from affected ducks in which they found necrotic foci in the liver, spleen and kidneys. Intramuscular inoculation of the reovirus caused mortality without clinical signs within 2 days and necrotic foci in the liver and spleen. Kuntz-Simon *et al.* (41) and Zhang *et al.* (77) suggested separate classification for Muscovy duck reoviruses based on sequencing of the σC encoding gene. No satisfactory vaccines have been marketed for these viruses but Kuntz-Simon *et al.* (42) have shown that a baculovirus-experssed σC alone, or with expressed σB appeared to be a good candidate for vaccination of ducks against the reovirus infection.

In a die-off of common eiders *(Somateria molissima)* in the Western Gulf of Finland Hollmen *et al.* (31) isolated a reovirus in cell cultures from Pekin duck but not chick embryo fibroblasts from several tissues of affected birds. High prevalence of neutralizing antibodies in flocks corresponded to mortalities, questioning their role as the cause.

The isolation of reoviruses from geese with Derzsy's disease has been reported (9), but their role is unknown as the condition is now known to be caused by infection with a parvovirus.

Serological evidence of reovirus infection in geese has been recorded in two reports. Kaleta *et al.* (34) detected neutralizing antibodies to a virus originating from Muscovy ducks and the standard chicken strain S1133 in sera of domestic geese *(Anser anser domesticus)*. Hlinak *et al.* (30) examined sera from bean geese *(Anser fabalis)* and white-fronted geese *(Anser albifrons)* in Germany. Avian reovirus antibodies were detected in 29% of blood samples, and there was no difference in seroprevalence between the two species. The authors indicate that although the role and significance of wild geese in the epidemiology of avian diseases remains to be determined, it is possible that they could be of some importance as reservoirs and carriers of some diseases of domestic poultry.

The first description of reovirus as a cause of arthritis in geese was that of Palya *et al.* (57). The disease was characterized by splenitis with miliary necrotic foci during the acute phase and

epicarditis, arthritis and tenosynocitis during the subacute/chronic phase. Clinical sings usually appeared at 2–3 weeks of age and persisted for 3–6 weeks. The reovirus was isolated from several organs and ths disease was reproduced in young goslings.

The sequence and structural similarities between the genome segment encoding σC in goose and and duck reoviruses lead Banvai *et al.*, (6) to suggest that these viruses belong to a species distinct from other established avian reovirus species within subgroup 2 of orthoreoviruses. Kunz-Simon *et al.*, (2002) and Zhang *et al.* (2006) have also suggested separate classification for Muscovy duck reoviruses based on sequencing of the σC encoding gene.

Reoviruses in Other Avian Species

McFerran *et al.* (46) isolated reoviruses from pigeons and Gough *et al.* (23) from diseased pigeons, pheasants, parrots, and other exotic avian species. Jones and Guneratne (33) isolated a reovirus from the feces of a zoo wedge-tailed eagle *(Aquila andax)*. This virus caused microscopic lesions of tenosynovitis in SPF chicks. All these reoviruses shared a common group antigen with chicken reoviruses, but their importance as pathogens in the host species was not determined.

Graham (24) isolated a reovirus from the liver of an African grey parrot submitted for necropsy with subcutaneous hemorrhages, multiple foci, and necrosis in the liver, spleen, bone marrow, intestinal lamina propria, airsacculitis, and epicarditis. Experimental inoculation of two African grey parrots with the isolate was fatal and reproduced the hemorrhages and necrotic lesions of the original condition. Sanchez -Cordon *et al.*, (67), describing disease in the same species involving 80% morbidity and 30% mortality considered that the reovirus they isolated probably triggered the subsequent herpesvirus (Pacheco's disease) and mycosis that caused the illness.

A virus associated with mortalities in American woodcock *(Scolopax minor)* was identified as a reovirus by Doherty *et al.* (15). A consistent necropsy finding was emaciation of the carcass. The authors considered that the reovirus infection was systemic and to be responsible for the deterioration in bodily condition of the birds. Transmission was thought to be by the fecal-oral route, but again, the true association with the disease was not confirmed.

An enteric disease in bobwhite quail *(Colinus virginianus),* which resulted in increased mortality in birds from 5 days to 5 weeks was described by Ritter *et al.* (61). A reovirus was isolated from the feces, and intestinal cryptosporidia were also present. In attempts to reproduce the condition experimentally, the reovirus induced subclinical infection, but the cryptosporidium caused changes resembling the natural disease. Infection of quail with both agents produced systemic infection (25).

An outbreak of disease in pheasants in Turkey attributed to reovirus infection was described by Mutlu *et al.* (52). Twenty-seven of a flock of 100 were affected between 3–5 months of age. In addition to being in poor condition, affected birds were short of breath, had greenish diarrhea, and died within a week. Pathological findings comprised fibrinous tracheitis, catarrhal inflammation of the gut, severe hepatic necrosis, and fibrinous perihepatitis. A reovirus was isolated from several organs, but whether this was the only agent involved was not investigated.

Curtis *et al.* (11) reported tenosynovitis in 6–7-week-old pheasants. *Staphylococcus aureus* and an avian reovirus related antigenitcally to strain S1133 were isolated from the swollen hock joints of lame birds. The association between the reoviruses as a cause of the disease was presumed but not confirmed.

Antibodies to avian reoviruses (and to other poultry pathogens) have been detected in ostriches *(Struthio camelus)* in Zimbabwe (8), rockhopper penguins (*Eudyptes chrysocomes*) in Argentina (37) and in bean geese (*Anser fabalis*) and white-fronted geese(*Anser albifrons*)in Germany (30). Again, the significance of these findings is unknown.

Conclusions

Reoviruses are very common among domestic poultry and are probably common in other avian species. They are viruses that are relatively easy to cultivate, and when they are looked for, serum antibodies are often found, so there is a temptation to implicate them as a cause of several conditions from which they have been isolated. Apart from tenosynovitis in chickens, where a clear relationship occurs between reovirus infection and the clinical disease, the role of reoviruses in avian disease is frequently unclear. In exotic birds, reports of reoviruses have been sporadic. There appears to be a wide range of pathogenicity among isolates, but most are probably harmless.

There may be differences in tissue tropism, although all appear to replicate in the gut, and pathogenic strains affect the liver. In most cases, the serologic or molecular relationship of reoviruses from exotic species to the tenosynovitis strains is unknown. Where exotic strains have been tested in chickens, a predilection exists for the hock joints or tendons, suggesting the potential for cross-species infection. However, exotic species have never been proven to be reservoirs of infection for domestic poultry.

Because of the inconsistency of disease associated with reoviruses in species other than the chicken, vaccines have not been developed.

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Chapter 12

Viral Enteric Infections

Introduction

Y. M. Saif

Our knowledge of enteric viruses has increased substantially in the last three decades. Several factors have contributed to this progress. The realization that pathogens other than bacteria must be an important component of the etiology of enteric disease has fueled the search for other infectious agents with specific emphasis on viruses. The availability of diagnostic tools was another incentive. Most useful among these tools was direct and immune electron microscopy.

The morphologic identification of these viruses by electron microscopy from gut contents paved the way for attempts to purify, cultivate, and develop diagnostic reagents and to further characterize these agents. Another technique that was used successfully earlier to identify some viruses in fecal samples is electropherotyping of genomic RNA. This technique is useful for detection and differentiation of double-stranded RNA viruses, such as rotaviruses and reoviruses.

A significant finding reported in the earlier studies was the presence of a variety of viral species that could be present in different combinations in the gastrointestinal (GI) tract of young commercial poultry. That necessitated attempts to separate these viruses to facilitate the study of their role in enteric disease. The availability of specific-pathogen-free (SPF) poultry was of major value in studies on these viruses. In the last decade, more diagnostic technologies such as reverse transcriptase/polymerase chain reaction (RT/PCR) became available and currently are used routinely in some laboratories to detect enteric viruses in gut contents. Removal of inhibitors from fecal material increased the utility of the test. In addition, the use of internal controls has improved the test specificity.

Most enteric viral infections occur in the first 3 weeks of life, but some could occur later. The clinical signs and lesions induced by the different viruses have similarities; hence, it is difficult to attribute a specific enteric disease to a given virus unless laboratory studies are initiated to identify the causative agent(s). In addition, the presence of different combinations of viruses could result in varied disease presentations. In general, high morbidity and low mortality exist when only one virus is detected, but mortality could be high when several viruses are present. An example of the significant economic impact of these combinations is the condition that was designated poult enteritis and mortality syndrome that was identified in the United States.

Different terms have been used in the literature to describe different conditions/syndromes of enteric disease. Unfortunately such descriptions are not instructive and confusing at best since these descriptions do not refer to specific etiology/ies. Since diagnostic tools are available for most enteric viral infections, it is preferable to designate these conditions as enteritis with reference when available to the specific infectious agents involved.

Diarrhea is a common manifestation of the disease, and the gastrointestinal (GI) tract is usually distended with gas and liquid contents. Different viruses replicate at different parts of the GI tract and at different sites on the villi. Epidemiologic studies indicated that these viruses do not persist for long in the birds. Unfortunately, many of the enteric viruses are uncultivable which hampers research and diagnostics.

No evidence of egg transmission of enteric viruses exists, and there are gaps in our knowledge of the epidemiology of the infections. Active immunity apparently plays a role in limiting the disease, but the benefits of passive immunity are limited to the first few days of life. No commercial vaccines are available for most of these infections.

Enteric viruses are commonly the cause of most of the primary insults to the GI tract of young poultry. This provides other agents, especially bacteria, with the milieu to replicate, attach, and penetrate, leading to further damage. It has been shown that during the course of several enteric viral infections, bacteria adhere and form a membrane on the surface of the villi. Counteracting secondary bacterial involvement is probably the reason for the reported effectiveness of antibiotic treatment of some cases of enteric disease initiated by viruses in young birds.

Most of the knowledge on enteric viruses were derived from studies on turkeys because of the economic significance of the disease caused by enteric viruses in commercial turkey poults. Lately, there has been renewed interest in studying enteric viral infections in young broilers because of the emergence of enteric disease as an economically significant problem in broiler operations.

The GI tract has the most extensive exposed surface in the body, and it is continually exposed to a variety of insults and

stimulations. In food animals, the integrity of the GI tract is of paramount importance. Efficient utilization of nutrients is dependent on a healthy GI tract, and this is especially true for the young of the species. Damage to the GI tract early in life could result in irreversible damage to the flock.

The progress made in the last three decades has been remarkable, but there remain gaps in our knowledge of enteric viruses. Further information that could lead to novel methods for control of these infections should be of significant impact.

Turkey Coronavirus Enteritis

J. S. Guy

Introduction

Turkey coronavirus (TCV) is the cause of an acute highly contagious enteric disease of turkeys characterized by depression, anorexia, diarrhea, and decreased weight gain. Bluecomb disease, mud fever, transmissible enteritis, and coronaviral enteritis are synonyms of TCV enteritis of turkeys.

History

In 1951, Peterson and Hymas (59) described an enteric disease of turkeys that had been observed for several years in Washington state and was known locally as mud fever. Later, the disease became known as bluecomb disease owing to clinical similarities with avian monocytosis (bluecomb disease of chickens). Severe economic losses were attributed to bluecomb disease in the United States and Canada in the 1950s and 1960s. Between 1951 and 1971, bluecomb disease was considered to be the most costly disease affecting Minnesota turkey production (50). Economic losses were attributed to increased mortality and weight loss in affected turkeys. In 1966, 23% of all mortality in Minnesota turkey flocks was attributed to bluecomb disease (58).

Efforts to identify the etiology of bluecomb disease extended over a 20-year period beginning in the early 1950s (58). Several different infectious agents were identified in affected turkeys, including reoviruses, enteroviruses, and *Campylobacter* spp.; however, experimental attempts to reproduce the disease with these agents were unsuccessful (20, 24, 74, 75, 82). In 1971, Adams and Hofstad (1) successfully propagated a virus from bluecomb disease-affected turkeys in embryonated chicken and turkey eggs, and experimentally reproduced the disease using this embryo-propagated virus. A coronavirus was identified as the etiology in 1973 (55, 65).

Eradication efforts were begun in Minnesota in the early 1970s and these efforts led to successful elimination of the virus from Minnesota turkey flocks by 1976 (58). After this time, TCV was identified only sporadically in turkey producing areas of North America. In recent years, TCV has been increasingly identified in North America as an important cause of enteric disease of turkeys. Turkey coronavirus also has been associated with a disease referred to as poult enteritis– mortality syndrome, a disease characterized by high mortality, severe growth depression and immune dysfunction (5).

Etiology

Classification

Turkey coronavirus is classified as a member of the Coronaviridae (23). The Coronaviridae comprise a large family of RNA-containing viruses that infect a wide variety of avian and mammalian species (69). The *Coronaviridae* is in the order *Nidovirales*, an order composed of viruses having linear, nonsegmented, positive-sense, single-stranded RNA genomes with similar genomic organization and nested sets of subgenomic mRNAs (13). The coronavirus genome consists of an RNA molecule that is approximately 30 kilobases in size (69). Coronaviruses possess four major structural proteins referred to as surface ("spike") glycoprotein (90–180 kilodaltons [kDa]), integral membrane protein (20–35 kDa), small envelope protein (12.5 kDa) and nucleocapsid protein (50–60 kDa) (69). In addition, some coronaviruses also contain a fifth major structural protein, the hemagglutinin-esterase protein (120–140kDa) (33, 69).

Coronaviruses have been subdivided into three major antigenic groups based on antigenic differences identified by serological analyses, and these findings have been substantiated by nucleotide sequence analyses (33, 69). Human coronavirus (HCV) (229E strain), porcine transmissible gastroenteritis virus, canine coronavirus and feline infectious peritonitis virus are members of group 1. HCV (OC43 strain), murine hepatitis virus, porcine hemagglutinating encephalomyelitis virus and bovine coronavirus (BCV) are members of group 2. Infectious bronchitis virus (IBV) and TCV comprise group 3 (11, 12, 14, 27).

Early antigenic analyses based on immune electron microscopy, hemagglutination inhibition, and virus neutralization assays indicated that TCV and IBV were antigenically distinct from each other and mammalian coronaviruses (15, 65). Based on these studies, IBV and TCV were classified as members of coronavirus antigenic groups 3 and 4, respectively (81). Subsequently, Dea *et al.* (17) provided evidence that TCV and BCV, a group 2 coronavirus, were closely related viruses based on a variety of antigenic and genomic analyses (17, 78, 79). However, more recent studies have demonstrated that TCV is antigenically and genetically closely related to IBV, a group 3 coronavirus (11, 12, 14, 27, 39, 40, 41). Turkey coronavirus was shown to be closely related to IBV based on antigenic analyses (28, 36, 39, 42) and these findings subsequently were substantiated by nucleotide sequence analyses (4, 6, 7, 14, 36, 39, 40, 41, 73). Several different studies have indicated a high degree of sequence identity between se-

quences of the integral membrane protein, nucleocapsid protein and polymerase (ORF1b) genes of TCV and IBV; only limited sequence identity was observed with the same sequences of mammalian coronaviruses (4, 6, 7, 14, 36, 40, 41, 73).

Laboratory studies have shown that BCV can replicate in intestinal tissues of inoculated turkey poults (18, 35). Dea *et al.* (18) demonstrated replication of two different BCV strains in intestines of experimentally infected turkeys, but infection failed to produce clinically apparent disease or intestinal lesions. In a recent study, Ismail *et al.* (35) demonstrated BCV replication in intestinal tissues of experimentally inoculated turkeys with development of mild clinical disease (diarrhea, body weight gain reduction, no mortality) and mild to moderate intestinal lesions. However, naturally occurring infection of turkeys with BCV has not been demonstrated.

Morphology

Coronaviruses are roughly spherical, pleomorphic, enveloped particles, with diameters of 60–200 nm (33, 69). They possess a characteristic surface structure composed of long (12–24 nm), widely spaced, club-shaped peplomers (Fig. 12.1). These peplomers give virions the distinctive appearance of a solar corona, hence the name coronavirus. Turkey coronavirus has been shown to have a density of 1.16–1.24 g/ml in sucrose (15).

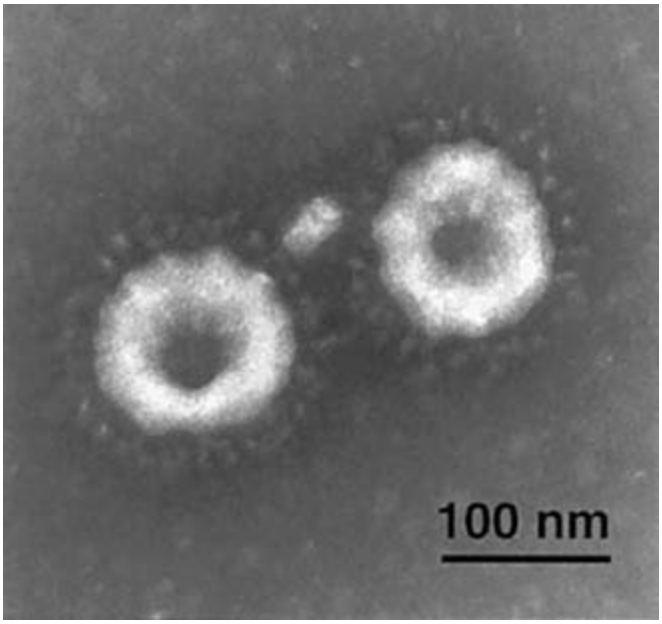
Chemical Composition

Only limited information is available regarding the chemical composition of TCV. Studies indicate the presence of a RNA genome (19), but little information is available regarding specific properties of TCV RNA. Turkey coronavirus RNA likely is similar to that of other coronaviruses: linear, nonsegmented, single-stranded and approximately 30 kilobases in size (12, 13). Nucleotide sequence analyses of TCV RNA have demonstrated that TCV, like IBV, possess surface ("spike") glycoprotein, integral membrane, and nucleocapsid proteins (6, 7, 14, 40, 41). However, the size and structural properties of these proteins have not been determined. No information is available regarding other TCV proteins.

Virus Replication

Based on immunofluorescence and immunoperoxidase staining procedures, TCV was shown to replicate primarily in enterocytes in the jejunum and ileum (3, 8, 56, 62), and epithelium of the bursa of Fabricius (28). Viral antigen in intestinal enterocytes was found predominately in enterocytes lining the upper one-half to two-thirds of intestinal villi (Fig. 12.2) (8, 28, 62). In the bursa of Fabricius, viral antigens are found in both follicular and interfollicular epithelium of the bursa of Fabricius (Fig. 12.3); viral antigens are not found in bursal lymphoid follicles. In inoculated embryos, virus replication occurs exclusively in intestinal epithelial cells (62); virus replication has not been detected in allantoic, yolk or amniotic membranes.

Thin-section electron microscopy of intestines from TCV-infected embryos and poults (3, 62) has shown that TCV replication occurs in the cytoplasm. Turkey coronavirus acquires its envelope by a process of budding through membranes of the endoplasmic reticulum and Golgi apparatus; virus particles accumulate in cisternae of the endoplasmic reticulum.



12.1. Negative contrast electron micrograph of turkey coronavirus.

Susceptibility to Chemical and Physical Agents

Turkey coronavirus was shown to be stable at pH 3 at 22°C for 30 min, and resistant to 50°C for 1 hr, even in the presence of 1 M magnesium sulfate (19). Chloroform treatment at 4°C for 10 min readily inactivated the virus.

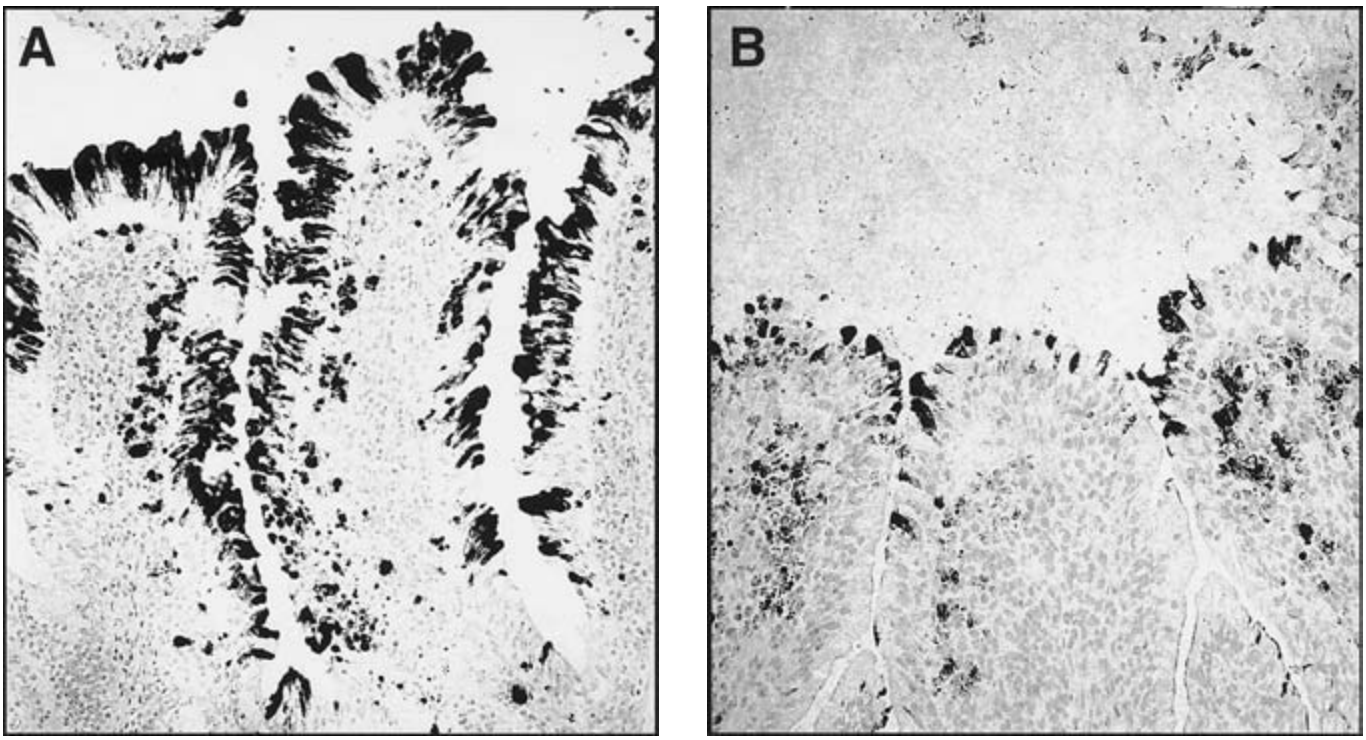
Turkey coronavirus was shown to remain viable in intestinal tissues stored at –20°C or lower for over 5 years. In Minnesota, TCV was shown to survive in buildings and ranges for extended periods of time even after turkeys were removed from these premises (50). Saponified cresol and formaldehyde were shown to be effective disinfectants for eliminating TCV from contaminated buildings (58).

Strain Classification

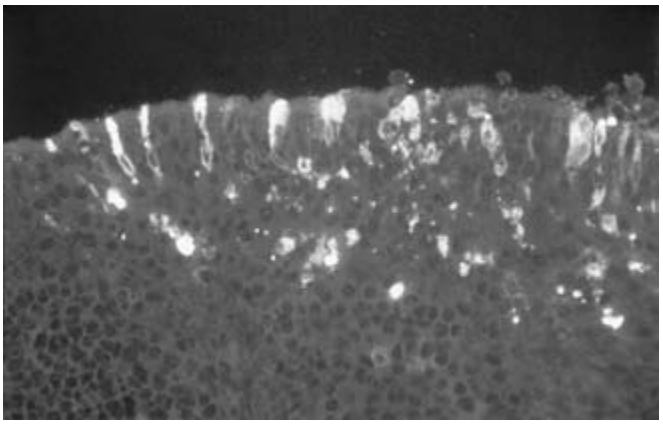
Recent studies indicate that TCV strains are antigenically and genetically closely related (6, 7, 28, 36, 39, 41). Antigenic analyses based on cross-protection studies, cross-immunofluorescence and enzyme-linked immunosorbent assays indicate close antigenic relationships between TCV isolates obtained from different geographical locations (28, 36, 39). Similarly, nucleotide sequence analyses of surface ("spike") glycoprotein gene, nucleocapsid gene and the 3′ untranslated region of different TCV isolates have demonstrated that these viruses are genetically very similar (6, 7, 36, 41). Studies have not been done to examine differences in virulence among different isolates of TCV.

Laboratory Host Systems

Turkey coronavirus strains can be propagated in embryonated chicken eggs (>16 days of incubation) and embryonated turkey



12.2. Immunoperoxidase staining of intestinal tissues from TCV-infected turkey. A. Cecum, 1 day PI. B. Ileum, 14 days PI. ×350.



12.3. Immunofluorescent staining of bursa of Fabricius of TCV-infected turkey, 2 days PI. Note that staining is localized to bursal epithelium. ×240.

eggs (>15 days of incubation) by inoculation of the amniotic cavity (1). In inoculated embryos, virus is recovered only from intestines and bursa of Fabricius (50).

Attempts to propagate TCV in a variety of avian and mammalian cell cultures generally have been unsuccessful (21, 50). Dea *et al.* (16) reported the cell culture adaptation and serial propagation of TCV using a human rectal adenocarcinoma (HRT) cell line; however, this has not been corroborated by other investigators (28, 35, 63).

Pathobiology and Epidemiology

Incidence and Distribution

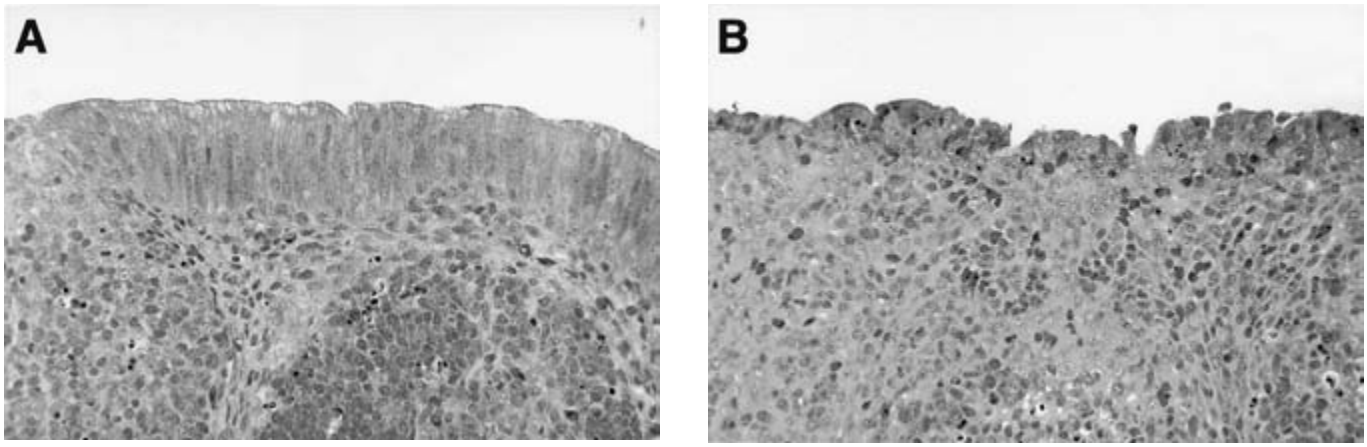
Turkey coronavirus has been identified in turkeys in the United States, Canada, Brazil, Italy, United Kingdom, and Australia (12, 14, 15, 50). The virus has been identified in most turkey producing regions of the United States.

Natural and Experimental Hosts

Turkey coronavirus affects turkeys of all ages; however, clinical disease most commonly is observed in young turkeys during the first few weeks of life. Turkeys are believed to be the only natural host for TCV. Pheasants, sea gulls, coturnix quail, and hamsters are refractory to infection (32, 50). Chickens once were believed to be refractory to TCV infection (60, 71); however, recent studies indicate otherwise (29, 37). In two separate studies, specific-pathogen-free chickens did not exhibit clinically apparent disease after experimental inoculation with TCV; however, susceptibility to TCV infection was demonstrated by seroconversion and detection of virus and viral antigens in intestinal tissues and bursa of Fabricius on days 2–8 postinoculation (PI) (29), and by detection of virus is intestinal contents on days 1–14 PI (37). Turkey coronavirus was not detected in other tissues including trachea, lung and kidneys (29).

Transmission, Carriers, Vectors

Turkey coronavirus is shed in feces of infected birds and spread horizontally through ingestion of feces and feces-contaminated materials. Experimental attempts to infect turkeys with ho-



12.4. Histopathologic changes observed in bursa of Fabricius of TCV-infected turkey. A. Sham-inoculated control. Note that epithelium consists of pseudostratified columnar epithelium. B. TCV-infected turkey, 4 days PI. Note epithelial cell necrosis and hyperplasia with heterophilic inflammation. ×240.

mogenates of liver, heart, spleen, kidney and pancreas of infected turkeys were unsuccessful. Turkeys were readily infected using filtered or unfiltered intestinal materials, or filtered homogenates of the bursa of Fabricius of infected turkeys (50, 53).

Turkey coronavirus generally spreads rapidly through a flock and from flock to flock on the same or neighboring farms. Mechanical movement of the virus may occur by people, equipment, vehicles and insects. Darkling beetle larvae and domestic house flies have been shown to be potential mechanical vectors of TCV (10, 80). Wild birds, rodents, and dogs also may serve as mechanical vectors. There is no evidence that TCV is egg transmitted; however, poults may become infected in the hatchery via contaminated personnel and fomites such as egg boxes from infected farms.

Turkey coronavirus is shed in droppings of turkeys for several weeks after recovery from clinical disease (8, 38). The virus was detected in intestinal contents of experimentally inoculated turkeys for up to 6 weeks PI by virus isolation, and up to 7 weeks PI by a reverse transcriptase-polymerase chain reaction (RT-PCR) procedure (8).

Incubation Period

The incubation period may vary from 1–5 days, but typically is 2–3 days.

Clinical Signs

In field cases, clinical signs occur suddenly, usually with high morbidity. Birds exhibit depression, anorexia, decreased water consumption, watery diarrhea, dehydration, hypothermia and weight loss. Droppings typically are green to brown, watery, frothy, and may contain mucus and urates. Flocks infected with TCV experience increased mortality, growth depression, and poor feed conversion compared with uninfected flocks (66). Mortality is variable in affected flocks; high mortality may occur depending on the age of the birds, concurrent infection, management practices and weather conditions.

Experimental studies using egg-adapted strains of TCV indicate that TCV infection results only in mild disease and moderate growth depression; mortality generally is negligible (30, 37, 51, 54, 63).

Turkey coronavirus infection of turkey breeder hens during production results in a rapid drop in egg production (50). Egg quality also is affected; hens produce eggs that lack normal pigmentation (white, chalky eggs).

Pathology

Gross

Gross lesions are seen primarily in intestines and bursa of Fabricius. Duodenum and jejunum generally are pale and flaccid; ceca are distended and filled with watery contents. Atrophy of the bursa of Fabricius may be observed. Emaciation and dehydration also may be observed in infected turkeys.

Microscopic

Microscopic lesions are observed in intestines and bursa of Fabricius of TCV infected turkeys. In intestines, microscopic lesions in experimentally infected turkeys consist of a decrease in villous length, increase in crypt depth, and decreased intestinal diameter (2, 25). The columnar epithelium of intestinal villi changes to a cuboidal epithelium and these cells exhibit a loss of microvilli. There is a decrease in number of goblet cells, separation of enterocytes from lamina propria, and infiltration of lamina propria with heterophils and lymphocytes. Epithelial repair is evident beginning at 5 days PI, and complete by 21 days PI (2, 25). By 5 days PI, columnar epithelium with microvilli begin to replace cuboidal cells, and goblet cells begin to reappear (25).

In the bursa of Fabricius, changes in epithelial cells are evident by 2 days PI and consist of epithelial necrosis and hyperplasia (30). The normal pseudostratified columnar epithelium of the bursa of Fabricius is replaced by a stratified squamous epithelium (Fig. 12.4). Intense heterophilic inflammation is observed

within and subjacent to the epithelium. Moderate lymphoid atrophy of bursal follicles is observed; however, as TCV antigens are not detected within lymphoid follicles, it is unlikely that TCV directly damages bursal lymphoid tissue. Lymphoid depletion in the bursa of Fabricius may occur secondary to damage of bursal epithelium or as a result of glucocorticoid release during infection. Epithelial repair was evident in the bursa of Fabricius by 10 days PI, with the appearance of areas of columnar epithelium replacing stratified squamous epithelium.

Ultrastructural

Ultrastructural changes in intestines of TCV-infected turkeys are confined to epithelial cells (3, 62). Ultrastructural changes include loss of microvilli, disruption of the terminal web region, degeneration of mitochondria, dilation of cisternae in the endoplasmic reticulum, increases in intracellular lipid, excessive sloughing of cells at villous tips, and shortening of villi. Coronavirus particles (80–140 nm in diamter) are observed within cisternae of the endoplasmic reticulum (62).

Pathogenesis of the Infectious Process

Turkey coronavirus replicates preferentially in enterocytes lining the apical portions of intestinal villi (30, 62) and in epithelium of the bursa of Fabricius (21). The site of intestinal TCV infection suggests that the virus may cause malabsorption, maldigestion and diarrhea in a manner similar to other enteric coronaviruses (46, 67). Malabsorption, maldigestion and diarrhea likely result from TCV-induced destruction of villous epithelium; however, it has been suggested that the virus may exert its effects in a more subtle manner through alterations in the physiology of these cells (62). Turkey coronavirus also may exert its effects by altering the normal intestinal flora (52). The intestinal flora of TCV-infected turkeys was characterized by increased numbers of putrefactive and lactose nonfermenting bacteria, and a simultaneous increase in lactobacilli.

Severe disease characterized by high mortality was a common feature of early descriptions of TCV infection (bluecomb disease) and early experimental studies using inocula composed of crude fecal/intestinal homogenates (50). More recent experimental studies using embryo-propagated TCV indicate that mortality due to TCV infection usually is negligible, at least under laboratory conditions (30, 37, 51, 54, 63). Weather conditions, management practices, crowding, and secondary infections may exacerbate the effects of TCV infection and result in increased losses. Antibiotics have been shown to reduce mortality in TCV-infected flocks, most likely because they control secondary bacterial infections (59, 70). Experimental studies with TCV and an enteropathogenic strain of *Escherichia coli* provide evidence suggesting an interaction between TCV and bacteria in the development of severe clinical disease (30, 54). In these experiments, young turkeys inoculated with only TCV developed moderate growth depression without significant mortality, and turkeys inoculated with only enteropathogenic *Escherichia coli* did not develop clinically apparent disease. However, turkeys dually inoculated with TCV and enteropathogenic *Escherichia coli* developed severe growth depression and high mortality.

Immunity

Active

Turkeys that recover from TCV infection are resistant to subsequent challenge (50, 61). Turkeys that survived experimental TCV infection at 4 days of age showed no clinical signs when challenged at 11 and 22 weeks of age (61). Field observations indicate that flocks that recover from TCV infection are resistant to subsequent infection (60).

The nature of protective immunity in recovered birds is not fully understood. Specific secretory IgA, humoral and T-cell mediated immunity have been demonstrated in recovered turkeys (33, 43, 44, 47, 48). Specific secretory IgA was shown to persist in intestinal secretions and bile of recovered turkeys for at least 6 months (47). In a recent study, TCV-specific IgA antibodies were measured in feces of TCV-infected turkeys using an enzyme-linked immunosorbent assay; these studies demonstrated that IgA antibody production peaked at 3–4 weeks postinoculation and disappeared at approximately 6 weeks postinoculation (44).

Passive

Poults passively immunized against TCV by subcutaneous inoculation of serum from immune birds were not protected from challenge (60). Poults from immune and nonimmune breeder hens were equally susceptible to TCV challenge (75).

Diagnosis

Isolation and Identification of Causative Agents

Diagnosis of TCV infection generally requires laboratory assistance as other enteric pathogens of turkeys may cause similar clinical signs and lesions. Laboratory diagnosis may be achieved based on virus isolation, electron microscopy, serology, or detection of viral antigens or viral RNA in intestinal tissues, bursa of Fabricius or intestinal contents.

Isolation of TCV may be accomplished by inoculation of embryonated chicken or turkey eggs (see Laboratory Host Systems) with suspensions of intestinal contents, dropping samples, or tissues (intestines, bursa of Fabricius) from suspect infected turkeys. Clinical samples should be homogenized in an appropriate diluent, such as minimal essential medium, clarified by centrifugation, and filtered through a 0.45 um filter. Embryonated chicken eggs (>16 days of incubation) or turkey eggs (>15 days of incubation) are inoculated by the amniotic route and returned to the incubator. Embryonated turkey eggs are the preferred substrate, as the relative sensitivity of chicken embryos to TCV has not been determined. After incubation for 2–5 days, the presence of TCV in embryo intestines is determined by immunohistochemical staining procedures. Immunohistochemical staining using either immunofluorescent antibody (FA) or immunoperoxidase (IP) procedures requires a virus-specific antiserum.

Diagnosis based on electron microscopy requires the identification of virus particles having typical coronavirus morphology. Coronaviruses must be distinguished from cell membrane fragments that may resemble coronaviruses; these cell membrane fragments commonly are identified in dropping samples from normal turkeys. Definitive identification of turkey coronavirus

may be accomplished by immune electron microscopy (64); however, this requires a source of virus-specific antisera.

Both direct and indirect FA procedures have been described for detection of TCV antigens in intestinal tissues and bursa of Fabricius of infected turkeys (8, 56, 62). The direct FA procedure is an excellent diagnostic approach with respect to simplicity and speed of diagnosis. However, sensitivity and specificity of this procedure are dependent upon the quality of the antiserum used to prepare the fluorochrome-conjugated antibody, and once this reagent is produced it has a relatively short shelf life. The direct FA procedure was shown to detect TCV antigens in experimentally infected turkeys from 1–28 days PI (56).

Indirect FA and indirect IP procedures have been described for detection of TCV antigens in tissues of infected turkeys (8); these procedures utilized TCV-specific monoclonal antibodies (MAB). These MAB-based immunohistochemical procedures detected TCV antigens in intestines and bursa of Fabricius of experimentally infected turkeys as early as 1 day PI, and as late as 42 days PI. They were shown to have high specificity (>92%), but low sensitivity (61–69%) compared with virus isolation. TCV-specific MABs represent an unlimited source of high quality antibody for TCV detection.

Reverse transcriptase polymerase chain reaction procedures (RT-PCR) have been developed for detection of TCV RNA in dropping samples and intestinal contents of infected turkeys (8, 77). These RT-PCR procedures have been shown to be highly sensitive and highly specific. In one study, RT-PCR was shown to detect TCV RNA in experimentally infected turkeys as early as 1 day PI and as late as 49 days PI; sensitivity and specificity of the RT-PCR procedure was 93% and 92%, respectively, compared with virus isolation (8). Additionally, multiplex RT-PCR procedures have been developed that allow simultaneous detection of TCV and other enteric viruses (45, 68, 72).

Serology

Detection of TCV-specific antibodies most commonly is accomplished using the indirect FA procedure. Antigen for this procedure consists of either frozen sections of TCV- infected embryo intestines (57) or epithelium exfoliated from bursae of Fabricius of infected turkeys (26). Frozen tissue sections are prepared from intestinal tissues of TCV-infected turkey embryos, 24–48 hours after inoculation with embryo-adapted TCV strains (57). Antigen preparation by this method is slow and labor intensive. However, an advantage of this serological method is that it allows discrimination of false positive staining based on determining the site of intestinal staining (i.e. TCV preferentially infects apical villous epithelium). Alternatively, antigen slides may be prepared using exfoliated epithelial cells collected from bursae of Fabricius of 2-week-old turkeys, 4 days after TCV inoculation (26). Bursae are harvested from infected turkeys, rinsed in cell culture media, and incubated at 4°C for 18–24 hours with gentle stirring to exfoliate epithelial cells. Cells are concentrated by low speed centrifugation and then spotted onto glass slides. TCV-specific antibodies may be detected in experimentally infected turkeys as early as 7 days PI using either of the indirect FA methods described above. Turkeys infected early in the brooder house have been shown to remain serologically positive throughout the growout period (34).

Enzyme-linked immunosorbent assays (ELISA) have been described for detection of TCV-specific antibodies in turkeys (31, 42). TCV-specific antibodies may be detected in turkey sera using commercially available, IBV-coated ELISA plates. In addition, a competitive ELISA (cELISA) was developed that utilizes a recombinant baculovirus-expressed TCV nucleocapsid protein and a biotin-labeled monoclonal antibody specific for TCV nucleocapsid protein (9, 31). The IBV ELISA and cELISA were both shown to be highly sensitive (>92) and highly specific (>96%) compared with the indirect FA procedure, and both tests cross-react with IBV antibodies (31, 42).

The IBV ELISA and cELISA do not discriminate between IBV- and TCV-specific antibodies. However, this is unlikely to be an impediment to specific detection of TCV infection in turkeys, as turkeys are not believed to be susceptible to IBV. IBV, like TCV and other coronaviruses, has a limited host range. Chickens and pheasants are the only known natural hosts for IBV; experimental attempts to infect turkeys with IBV have not been successful (27, 31).

Differential Diagnosis

Enteric disease caused by TCV must be distinguished from other enteric diseases of turkeys, particularly those caused by other viruses, bacteria and protozoa.

Intervention Strategies

Management Procedures

Prevention is the preferred method for controlling TCV. Turkeys infected with TCV have been shown to shed virus in feces for prolonged periods of time after recovery (8, 38, 56); these turkeys, their feces, and the materials that their feces contact are potential sources of infection for other susceptible turkeys. Feces from TCV-infected turkeys can be carried on a variety of fomites including clothing, boots, equipment, feathers and trucks. Other potential vectors such as wild birds, rodents, dogs and flies also may be involved in transmission from infected to susceptible flocks. Sound biosecurity measures must be instituted to prevent introduction of TCV via potentially contaminated personnel, fomites, animal and insect vectors, and infected turkeys.

Elimination of TCV from contaminated premises may be accomplished by depopulation followed by thorough cleaning and disinfection of houses and equipment (58). Following cleaning and disinfection procedures, premises should remain free of birds for a minimum of 3–4 weeks.

Vaccination

No licensed vaccine is available.

Treatment

At present, there is no specific treatment for TCV enteritis. Antibiotic treatment has been shown to reduce mortality, most likely by controlling secondary bacterial infections (59, 50, 70). No beneficial effect was observed when glucose, electrolytes, or calf milk replacer was added to drinking water (22).

Management procedures that have been effective in reducing mortality include raising brooder house temperatures and avoiding crowded conditions.

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Rotavirus Infections

M. S. McNulty and D. L. Reynolds

Introduction

Rotaviruses are now established as a major cause of enteritis and diarrhea in a wide range of mammalian species, including humans (50,111). Rotavirus infection in avian species was first reported in 1977 by Bergeland *et al.* (7), who found particles morphologically indistinguishable from rotaviruses in intestinal contents of poults with watery droppings and increased mortality. Since then, it has become apparent that rotaviruses infect many species of domesticated birds.

As in mammals, rotavirus infection in avian species is frequently associated with outbreaks of diarrhea. The economic significance of rotaviral enteritis to the poultry industry has not yet been defined, but by analogy with the situation in mammals, it is likely to be significant. Some mammalian rotaviruses have limited ability to infect other mammalian species, and rotaviruses from turkeys and pheasants can infect chickens (119). There is a report of the isolation of an avianlike group A rotavirus from a calf with diarrhea (10); gene sequences and other data provide convincing evidence that this virus originated from pigeons (8, 89, 90, 91). Also, mammalian-like group A rotaviruses have been detected in chickens with diarrhea (115), and experimental infection of suckling mice with avian rotaviruses has been described (74). However, interspecies transmission of rotaviruses between birds and to mammals and vice versa is probably rare. Avian rotaviruses have no known public health significance.

In this section, the term *rotavirus* includes those viruses formerly described as atypical rotaviruses, pararotaviruses, antigenically distinct rotaviruses and rotaviruslike viruses.

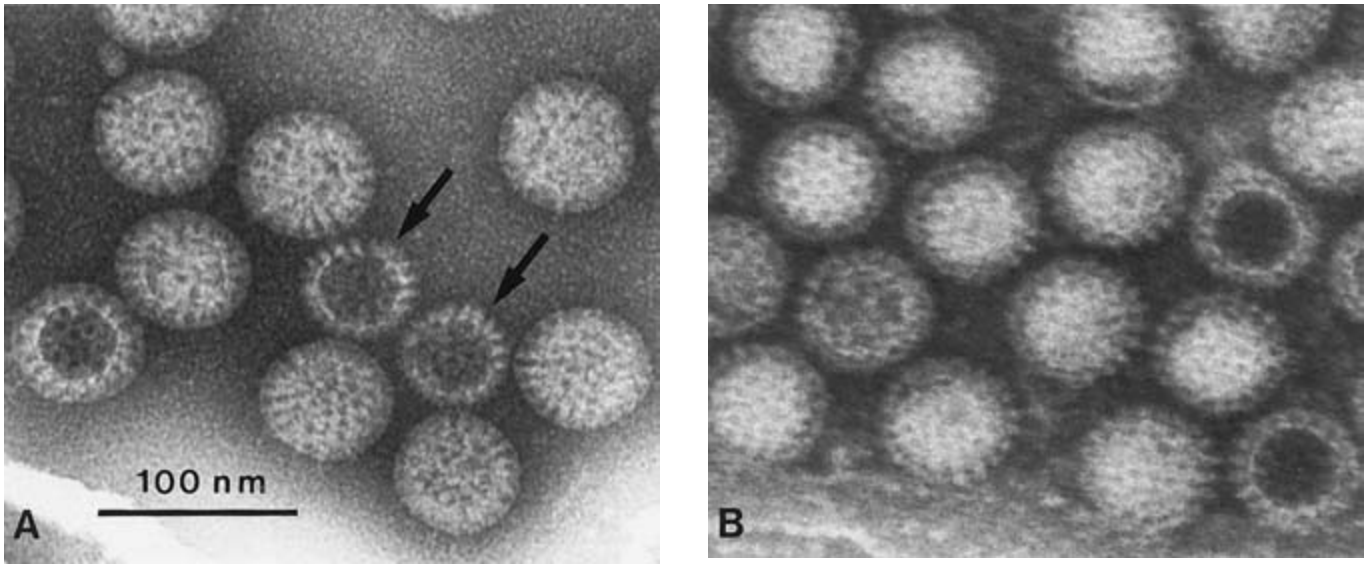
Etiology

Classification

Rotaviruses are classified as a genus in the family Reoviridae. This family contains 12 genera, members of which infect vertebrates, invertebrates, plants and fungi. Rotaviruses infect only vertebrates and are transmitted by the fecal-oral route. They differ in morphology from other genera within the family. Rotaviruses possess 11 segments of double-stranded RNA (85). Their morphogenesis involves the temporary acquisition of a lipid envelope and the deposition of viral-coded glycoprotein in the endoplasmic reticulum (36, 65). Rotaviruses readily undergo genetic reassortment (i.e., when two different rotaviruses belonging to the same serogroup infect the same cell, hybrid viruses containing mixtures of the genome segments from each parent are generated).

Morphology

Intact rotavirus virions appear to be approximately 70–75 nm in diameter when visualized by negative contrast electron microscopy. They are composed of 3 concentric protein layers, comprising a core about 50 nm in diameter, enclosed within a double-shelled capsid made up of inner and outer shells. They have been described as reoviruslike but can be distinguished from reoviruses by their more clearly defined smooth outer edge (Fig. 12.5). Some negatively stained intact rotavirus particles resemble a wheel with short spokes radiating from a wide hub, hence the derivation of the name rotavirus from the Latin word *rota*, which means wheel. The outer capsid shell may be lost, producing noninfectious or poorly infectious so-called single-shelled particles



12.5. A. Rotavirus particles in chicken feces showing intact particles with smooth outer edge and particles with serrated edges (arrows), lacking outer capsid shell. B. Reovirus isolated from guinea fowl feces. Intact rotavirus and reovirus particles can be differentiated by the more distinct, smooth outer margin of rotavirus. Methylamine tungstate stain.

(9) that resemble orbiviruses and are about 10 nm smaller than intact double-shelled virions (Fig. 12.5). Intact and single-shelled particles of turkey rotavirus had densities in cesium chloride of 1.34 and 1.36 g/mL respectively (48). Intact and single-shelled group D rotavirus particles from a pheasant were reported to be larger, at 80 nm and 70 nm, respectively, than those of an avian group A rotavirus with densities of 1.347 and 1.365 g/mL, respectively (16). Advances in our understanding of the structure of the rotavirus virion have revealed that the so-called single-shelled particles are in fact double-layered. Similarly, intact virions, previously referred to as double-shelled particles, are triple layered.

Both inner and outer capsids have a T = 13(*l*) icosahedral surface lattice, with 132 channels spanning both capsid shells and extending inwards from the virion surface to the core; 60 short spikes extend 12 nm from the smooth surface of the outer capsid shell, so the mature infectious virion has an overall actual diameter of approximately 100 nm (65, 85).

Chemical Composition

Like their mammalian counterparts, avian rotaviruses possess a double-stranded RNA genome consisting of 11 segments ranging from about 0.2×10^6 to 2.1×10^6 in molecular weight (1, 6, 12, 18, 21, 24, 26, 27, 28, 45, 46, 53, 54, 55, 61, 64, 68, 69, 86, 87, 93, 102, 103, 104,105, 106, 107, 108, 109, 110, 114, 118).

Ten major virus polypeptides were detected in MA104 cells infected with a turkey rotavirus. Three polypeptides, designated VP1, VP2, and VP6, with approximate molecular weights of 125 kD, 100 kD, and 45 kD were associated with particles lacking the outer capsid. Polypeptides VP3, VP4, VP5s, and VP7, with molecular weights of 90 kD, 88 kD, 54–55 kD, and 37 kD, formed part of the outer capsid shell. The 37-kD polypeptide was glycosylated, and two nonstructural polypeptides (30 kD and 28 kD) were also identified as glycoproteins (47).

These results were similar but not identical to those obtained with the prototype mammalian rotavirus, SA-11. Four polypeptides, designated VP1, VP2, VP3, and VP6, with approximate apparent molecular weights of 125 kD, 94 kD, 88 kD, and 41 kD, respectively, are present in SA-11 particles lacking the outer capsid shell; VP1, VP2, and VP3 comprise the core, and VP6 is the only protein in the inner capsid shell. The outer capsid shell of SA-11 is composed of 2 polypeptides, designated VP4 and VP7 (molecular weights 88 kD and 38 kD, respectively); VP7 is glycoslysated. Proteolytic cleavage of VP4 to produce VP5* (60 kD) and VP8* (28 kD) results in enhancement of viral infectivity. There are 6 SA-11 rotavirus nonstructural proteins (19).

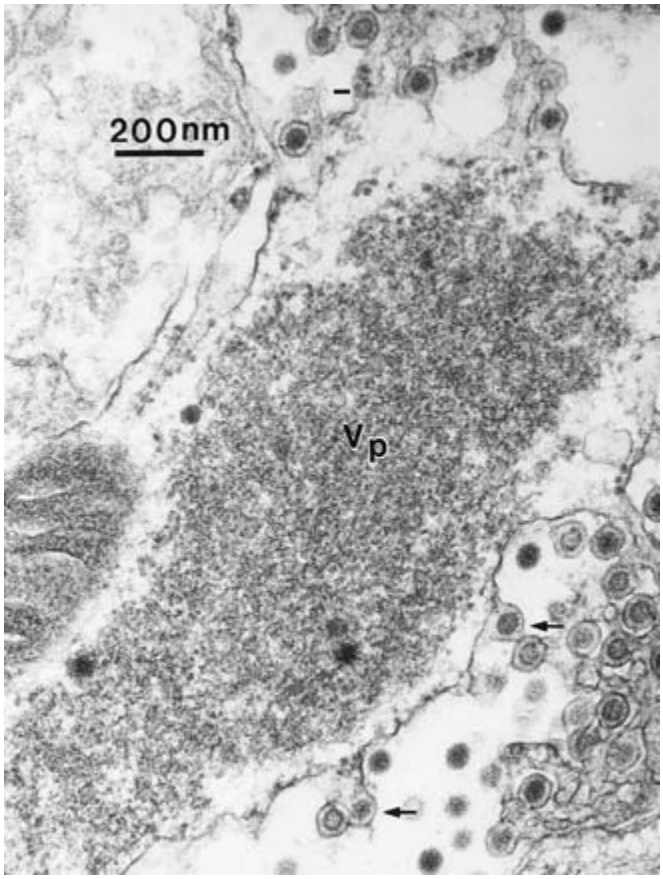
Brüssow *et al.* (11) identified putative VP1, VP2, VP3, V5*, VP6, and VP7 in purified, intact virions of rotavirus 993/83, an avianlike group A rotavirus isolated from the feces of a calf with diarrhea (10, 91) and of pigeon rotavirus PO-13, isolated in Japan (69). VP5* and VP7 were removed by EDTA treatment, indicating that they were present in the outer capsid shell (11).

The coding assignments and properties of the proteins encoded in each of the 11 genome segments of SA-11 rotavirus have been established, and the entire genome has been sequenced; this information is also available for a number of other mammalian rotaviruses (19, 85). Similarly, the complete nucleotide sequence of the avian rotavirus PO-13 pigeon isolate has been determined (40, 42). Also, sequence data and comparisons with their mammalian counterparts are available for the genes coding for VP3 (13), VP6 (18, 41, 42, 88, 90), VP7 (51, 79, 89), VP8* (91) and NSP4 (73) from a number of other avian group A rotaviruses.

Virus Replication

The replication of rotavirus has recently been reviewed in detail (19). Only those features of the morphogenesis of avian rotavirus likely to be of interest to avian pathologists are described here.

The morphogenesis of turkey, chicken, and pheasant rotaviruses has been investigated by thin-section electron microscopy (20, 34, 55, 61, 69). Virus replication occurs in the cytoplasm. Both in cell cultures and *in vivo*, virus cores are formed within granular matrices of viral precursor material (viroplasm) that lies free in the cytoplasm. Developing virus particles are liberated into dilated cisternae of rough endoplasmic reticulum. Some particles appear to bud through ribosome-free areas of endoplasmic reticulum, acquiring an envelope in the process (Fig. 12.6). The envelope is lost from particles in the interior of the endoplasmic reticulum and is replaced by a thin layer of protein that comprises the outer capsid shell. Virus is released by cell lysis.



12.6. Electron micrograph of chicken embryo liver cell culture 48 hours postinfection with turkey rotavirus. Part of the cytoplasm of an infected cell is shown, with viroplasm (Vp) containing virus cores and virus particles gaining envelopes by budding (arrows) from rough endoplasmic reticulum and from type 2 inclusion material. Nonenveloped virus particles are also present.

Susceptibility to Chemical and Physical Agents

Little published information exists about susceptibility of avian rotaviruses to chemical and physical inactivation. Two isolates of turkey rotavirus were stable to treatment with chloroform for 30 minutes and to pH 3 for 2 hours. Heating at 56°C for 30 minutes decreased infectivity of both viruses 100-fold, both in the presence and absence of magnesium ions (48). A duck rotavirus had similar resistance to inactivation with chloroform and acid but was less stable to heating in the presence of magnesium ions (100). Similarly, a pigeon rotavirus was stable to ether, chloroform, and sodium deoxycholate treatment (69). Glutaraldehyde had greater inactivating capacity against avian rotavirus than sodium hypochlorite or iodophor disinfectants (71).

Strain Classification

Antigenicity

Characterization of mammalian rotaviruses has shown that the most important antigens of the virus are those proteins that make up the inner and outer layers of the capsid (i.e., VP6, VP4, and VP7), although other proteins also contribute to the antigenicity of the virus. Rotaviruses have three important antigenic specificities, namely group, subgroup, and serotype (19, 50).

The vast majority of mammalian rotaviruses share a group antigen, demonstrable by tests such as ELISA, immune electron microscopy, complement fixation, and immunofluorescence. These have been termed group A or conventional rotaviruses to distinguish them from rotaviruses that were formerly termed atypical rotaviruses, which do not possess this antigen. This group reactivity resides predominantly in VP6, the protein that makes up the inner capsid shell; VP6 is also the major structural component of virions. Other terms for atypical rotaviruses include nongroup A rotaviruses, pararotaviruses, antigenically dis-

tinct rotaviruses, and rotavirus-like viruses. Atypical rotaviruses have been further divided into groups B, C, D, E, F, and G, based on possession of different group antigens and by terminal fingerprinting analysis of viral RNA (8, 36, 83, 84, 92).

Group A rotaviruses have been isolated from mammals and birds, but so far groups B, C, and E have been found only in mammals, and groups D, F, and G have been detected only in birds (36, 65).

Some avian rotaviruses show an antigenic relationship with mammalian group A rotaviruses by cross-immunofluorescence using hyperimmune or convalescent antisera (61, 64, 108, 118). Those avian rotaviruses antigenically related to mammalian group A rotaviruses are referred to as avian group A rotaviruses. This relationship originally was assumed to occur through simple sharing of the mammalian rotavirus group A antigen, but studies using monoclonal antibodies suggest a more complex situation. Some monoclonal antibodies, specific for group A avian rotavirus VP6, reacted with all mammalian and avian group A rotaviruses tested, and others recognized only avian group A rotaviruses (49, 70). Conversely, other monoclonal antibodies that recognized mammalian group A rotaviruses did not recognize avian group A rotaviruses (22, 30, 38). Thus, it appears that epitopes on VP6 of group A avian and mammalian rotaviruses exist, which are distinct from an antigenic determinant common to all group A rotaviruses.

Nucleotide sequencing and mapping of antigenic sites have provided evidence that the authentic group antigen common to mammalian and avian group A rotaviruses is located at amino acid residues 134–142 of PO-13 pigeon rotavirus VP6. In addition, another antigenic site shared by all mammalian and avian rotaviruses tested, with the exception of the Ch 1 chicken isolate, was present at amino acid residues 45–65 (39, 41). Interestingly, while VP6 from avian rotaviruses showed a low degree of nucleotide and deduced amino acid (approximately 70–75%) sequence homology with mammalian rotaviruses, Ch 1 VP6 sequences showed more than 13% amino acid differences compared to sequences from 2 pigeon and 2 turkey isolates (40, 88).

In addition to group specific antigenic domains, some domains on VP6 also mediate subgroup specificity. This has proven a useful epidemiological marker, particularly for human rotaviruses. Mammalian group A rotaviruses can be classified into subgroups I, II, I and II combined, or neither I nor II. Initial evidence suggested that group A avian rotaviruses belonged to neither mammalian rotavirus subgroup (22, 38, 49, 104), but more recent work has shown that avian rotaviruses from pigeons, turkeys, and chickens react with subgroup 1-specific group A monoclonal antibodies (70). However, additional studies are required to determine whether these avian group A rotaviruses are genuinely subtype I viruses.

In addition to group A avian rotaviruses, 3 other antigenically distinct groups of rotavirus have been identified by cross-immunofluorence in chickens (61, 64). The prototype viruses of these groups, the 132, A4, and 555 isolates have been classified as groups D, F, and G, respectively (8, 36, 84, 92). So far, groups D, F, and G have been identified only in avian species. Rotaviruslike viruses of turkeys (93, 105, 107) are related antigenically by cross-immunofluorescence to the 132 chicken rotavirus isolate (56) and should also be regarded as group D rotaviruses. Similarly, a rotaviruslike virus of pheasants has also been classified as a group D rotavirus (16). An avian group antigenically distinct from groups A and D, designated atypical rotavirus, has been identified in turkeys in the United States (105).

Viruses belonging to groups A–E are classified as belonging to different species in the genus *Rotavirus*, designated *Rotavirus A–E*, with viruses from groups F and G categorized as tentative additional species in the genus (65,85).

Serotype specificity in group A mammalian rotaviruses resides predominantly in VP7, a glycoprotein that constitutes most of the outer capsid shell and that, next to VP6, is the most abundant protein in the virus. Serotype specificity mediated by VP7 is termed G serotype. However VP4, the spike component of the outer capsid, also contributes to serotype specificity, and such specificity is termed P serotype. At least 15 G serotypes and 14 P serotypes have been recognized, based on virus neutralization tests using hyperimmune polyclonal antisera or monoclonal antibodies (85). Monoclonal antibody-based ELISAs are also used to determine G serotype (50). The genes encoding VP7 and VP4 can segregate independently during genetic reassortment. P serotyping has been hampered by a lack of readily available typing antibodies. However, it has been found that genotypes of VP4, as defined by nucleic acid hybridization and sequence data, correlate well with serotypes, so that genotyping can be used as a surrogate for serotyping (50).

Limited information is available about avian rotavirus serotypes. Using a fluorescent focus-neutralization test, 3 serotypes were identified in a collection of 6 turkey and 2 chicken isolates of group A avian rotavirus (62). With the use of a more sensitive plaque-reduction test, however, two of the viruses classified as different serotypes had a prime strain relationship and were classified in serotype G7 (38). So far, only avian rotaviruses have been assigned to this serotype. To date, there is no information about P serotypes of avian rotavirus. However, the limited sequence data relating to the genes encoding avian VP7 (51, 89) and VP8* (91), which possesses the major antigenic site(s) for P serotype specificity, indicate that diversity of both G and P serotypes exists in avian group A rotaviruses. There is no information about serotypic variation in nongroup A rotaviruses; however, it is likely that different serotypes exist in viruses belonging to groups B, C, D, E, and F.

Some group A and group D avian rotaviruses agglutinate a range of avian and mammalian erythrocytes (16, 32, 48, 69). In mammalian group A rotaviruses hemagglutination has been shown to be due to VP8* (50). Hemagglutination and hemagglutination-inhibition tests may also provide a means of strain classification.

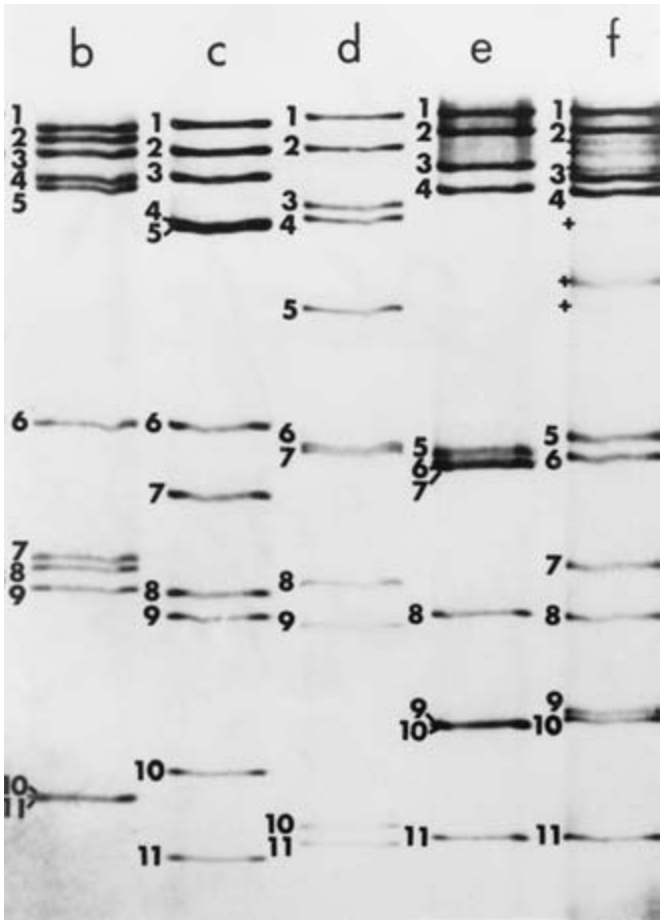
Genetic

Analysis of the pattern of migration of genome segments, especially segment 5; the triplet consisting of segments 7, 8, and 9;

and the doublet of segments 10 and 11, following polyacrylamide gel electrophoresis, has been extremely useful, both in preliminary characterization of avian rotaviruses and in investigating their epidemiology. An important advantage of this technique is that it does not require isolation and propagation of virus in cell cultures but can be carried out on virus in intestinal contents or feces. Five major types of RNA profiles, termed *electropherogroups*, were recognized when turkey and chicken rotavirus RNAs were electrophoresed (109) (Fig. 12.7). Rotaviruses belonging to electropherogroups 1, 2, 3, and 4 were detected in chickens, and electropherogroups 1, 2, 3, and 5 were found in turkeys.

Interestingly, 4 representative isolates, each belonging to different chicken electropherogroups, were also found to belong to different serogroups (64). Furthermore, turkey and pheasant group D rotaviruses from the United States have a similar pattern of migration of RNA segments to group D chicken rotavirus (16, 61, 64, 105, 109). This suggests that electropherogrouping may be a useful indicator of group antigenic differences. The existence of a fifth electropherogroup in avian species raises the possibility that an additional serogroup exists. Disruption of the triplet consisting of segments 7, 8, and 9 found in group A rotaviruses appears to be a useful marker for nongroup A rotaviruses. It will be interesting to see whether turkey viruses from the United States with genome profiles similar to those of electropherogroup 3 (45, 105) (i.e., so-called atypical rotaviruses) and chicken rotaviruses from Argentina (6) are antigenically related to group F chicken rotaviruses with similar profiles from the United Kingdom.

Within each electropherogroup, minor variations, termed *electropherotypes*, were described in turkey and chicken rotaviruses from the United Kingdom (109). Similar variations have also been described in turkey rotaviruses in the United States (45, 106). These variations may be useful for classifying rotavirus strains, although minor electrophoretic differences do not necessarily imply serotypic differences (48).



12.7. Genome profiles following electrophoresis of rotavirus RNAs in 5% polyacrylamide gel, showing profiles typical of avian electropherogroups 1 (lane b), 2 (lane c), 3 (lane d), 4 (lane f), and 5 (lane e). Genome segments are numbered 1–11; + indicates unidentified contaminating bands. (*Avian Pathology*)

Laboratory Host Systems

Isolations of rotaviruses from the feces or intestinal contents of turkeys, chickens, pheasants, ducks, and pigeons have been made in primary chick kidney and chick embryo liver cell cultures (3, 26, 60, 61, 62, 100, 118). Although a chicken rotavirus grew better in chick kidney cells than in a continuous line of fetal rhesus monkey kidney cells (MA104) (75), primary isolation of turkey (46, 107), pheasant (20), and pigeon (69) rotaviruses has also been achieved in MA104 cells. The pigeon isolate also replicated to higher titer in MDBK cells than in chick embryo kidney cell cultures (69). Some group A rotaviruses can infect both nonstimulated avian splenic lymphocytes and transformed avian lymphoblastoid cell lines (96).

Serial propagation of rotaviruses in cell culture usually requires trypsin treatment of virus inoculum. Proteolytic cleavage of VP4 into VP8* and VP5* potentiates *in vitro* growth by enhancing viral infectivity and penetration of the virus into cells (19, 50). Most isolates are noncytopathic on primary isolation; several passages in cell cultures are required before a cytopathic effect is seen. With the exception of the 132 chicken rotavirus isolate (61) and possibly isolates made from turkeys in France (3) and ducks in Japan (100), avian rotaviruses isolated and serially propagated to date in cell cultures have all been group A avian rotaviruses.

A rotavirus from lovebirds was lethal for chick embryos following yolk sac inoculation. Passage of the virus in 6–8-day-old embryos resulted in death 4–6 days after inoculation (24). Similarly, group A rotaviruses from turkey poults were isolated in chick embryos following yolk sac inoculation. Dead embryos were hemorrhagic and stunted with no other visible lesions (12). So far, no information is available concerning replication of other avian rotaviruses in embryos.

Accounts of experimental propagation of avian rotaviruses in their natural hosts are numerous (16, 20, 34, 57, 69, 81, 82, 119, 120, 121, 122). Some group A avian rotaviruses are also capable of infecting avian species other than their natural hosts (118, 119,

120, 122). In contrast, virus excretion was not detected in young chickens, turkeys, and partridges experimentally infected with an atypical rotavirus from pheasants (28).

Pathogenicity

Rotaviruses cause enteritis and diarrheal disease in avian and mammalian species. However, although rotavirus infections in avian species may be associated with outbreaks of enteric disease, subclinical infections are also common. In an extensive survey of turkey flocks with enteric disease and healthy turkey flocks in the United States, group A rotaviruses were detected slightly more commonly in healthy than diseased flocks; whereas rotaviruslike viruses were found far less frequently in healthy than in diseased flocks (86). A French study reported that 48.4% and 50% of chicks with diarrhea in 2000 and 2001 respectively were infected with rotavirus, which was found in only 20.2% and 18% of healthy chicks in the same years (94).

It has been established that mammalian rotaviruses can vary in virulence (19, 50). However, to date, no direct evidence shows that avian rotaviruses vary in virulence.

In theory, variations in virulence could arise through genetic reassortment. Genetic reassortment involving mammalian rotaviruses belonging to the same group, but not between viruses from different groups, has been described (19). *In vitro* reassortment has been documented between group A turkey and simian rotaviruses, producing a reassortant with the gene coding for VP4 originating from the simian parent and the remaining 10 genes coming from the avian rotavirus parent (52). However, if reassortment is important in generating new rotaviruses in avian species under field conditions, it is much more likely to occur between avian viruses than between avian and mammalian viruses.

Pathobiology and Epidemiology

Incidence and Distribution

Rotaviral enteritis has been described in poults in the United Kingdom (37, 60, 63), United States (7, 12, 93, 118), and France (2). Rotavirus isolation from, or detection in, chicken feces has been documented in Argentina (6), Belgium (66), Brazil (1), China (114), Cuba (21), Germany (18), India (68), the United Kingdom (59, 61, 62), the United States (118), and the former Soviet Union (4). Rotaviral antibody has been reported in chickens in Japan (95, 101), ducks in the United Kingdom (60), and pigeons in Belgium (113). Rotavirus was detected in the feces of guinea fowl with transmissible enteritis in Italy, but its etiologic role is uncertain (81). Rotaviruses have been found in feces of diseased pheasant chicks in Italy (20), the United Kingdom (27, 28), and the United States (87, 118). Rotaviruses were isolated from, or detected in, feces of clinically normal ducks in Japan (100) and the United Kingdom (112), apparently normal feral pigeons in Japan (69), diseased racing pigeons in the United Kingdom (26), diseased partridges in Italy (80) and the United Kingdom (23), diseased partridges and Japanese quail in Italy (80), a wild bird (*Melanitta fusca)* in Japan (102), and diseased ratites in South Africa (17) and the United States (35). A chicken embryo lethal rotavirus was isolated from the liver and small intestine of a diseased lovebird in England (24). This evidence indicates that rotaviruses have a worldwide distribution in a wide variety of avian species.

Natural and Experimental Hosts

As discussed previously, turkeys, chickens, pheasants, partridges, ducks, guinea fowl, pigeons, and lovebirds naturally are infected with rotaviruses, and some have been experimentally infected. Most naturally occurring infections in turkeys, chickens, pheasants, partridges, and ducks involve birds <6 weeks old. Paradoxically, older chickens (56–119 days) and turkeys (112 days) were more susceptible to experimental infection than birds in the first few weeks of life (119, 121). This observation is interesting; however, its relevance to the field situation is questionable because available evidence indicates that most turkeys and chickens will have been infected, and presumably will have developed some immunity, well before this age. Lack of age resistance to infection, however, is illustrated by an outbreak of diarrhea associated with rotavirus infection in commercial laying hens between 32 and 92 weeks of age (43).

Longitudinal surveys have shown that flocks of broilers and turkeys frequently experience simultaneous or sequential infections with different rotavirus electropherogroups (64, 86, 108, 109).

Some avian rotaviruses have been experimentally propagated in suckling mice (74).

Transmission, Carriers, and Vectors

Rotaviruses are excreted in avian feces in very large numbers (121). No information is available concerning survival of avian rotaviruses in feces, but, by extrapolation from mammalian rotaviruses, environmental contamination is likely to be persistent. Horizontal transmission occurs readily between birds in direct and indirect contact. Evidence demonstrates that larvae of the darkling beetle acts as a mechanical vector for turkey rotaviruses (15). Egg transmission of rotaviruses has not been demonstrated, but rotavirus detection in 3-day-old turkey poults prompted speculation that transmission occurs either in or on the egg (108). No evidence exists for a carrier state in birds.

Incubation Period, Clinical Signs, Morbidity, and Mortality

Both the incubation period and the course of the disease are short. In experimentally infected turkeys, watery-to-soft droppings were passed 2–5 days postinfection. Orange-tinged mucus was observed in soft feces. Rotavirus infection caused significant impairment of D-xylose absorption from the intestinal tract at 2 and 4 days postinfection. Inoculated turkeys were depressed with loss of appetite between 1 and 5 days postinfection. Pasting of the vents of experimentally infected poults has also been described (33, 119, 121). In the majority of studies, no mortality occurred in experimentally infected turkeys or chickens, but in one series of experiments, 3 of 23 poults inoculated at 2 days of age died (98). Mild (57) or no clinical signs (67, 119) were observed following experimental infection of chickens. When signs occurred, their onset coincided with peak virus excretion about 3 days

postinfection. Chicks had mild diarrhea (61) or passed increased quantities of cecal droppings (57). Laying hens experimentally infected with rotavirus showed a drop in egg production 4–9 days postinfection (121). Rotavirus was detected in feces of experimentally infected chickens and turkeys from 24 hours postinfection, and in some birds, excretion continued for more than 16 days (57, 119, 120, 121).

Under field conditions, clinical signs associated with rotavirus infection in broilers have varied from subclinical infections to outbreaks of diarrhea severe enough to warrant attention to the litter, with associated dehydration, poor weight gains, and increased mortality (1, 6, 60, 62). In poults, variations in severity of clinical signs have also been observed, including a very mild scour in the first week of life, which caused mortality only if vent pecking occurred (37); a more severe disease in 12–21-day-old poults characterized by restlessness, litter eating, and watery droppings with mortality between about 4 and 7% (7); and profuse scouring in 2–5-week-old poults (59), with affected birds huddling together, mortalities from suffocation, and stunting of survivors. In other outbreaks, predominant signs have been diarrhea and wet litter.

Pheasant chicks 2–3 weeks old in the United States had diarrhea and increased mortality associated with rotavirus infection (87). In the United Kingdom, rotavirus infection was associated with stunting and increased mortality in pheasant chicks in the first week of life (27, 28). Six of twenty 2-day-old pheasants inoculated with intestinal contents containing rotaviruses from naturally occurring cases died 5–6 days postinfection (28); a high mortality rate was also observed in pheasant chicks inoculated with a group D rotavirus (34). In Italy, infected pheasants between 6 and 40 days of age showed depression, drooping wings, yellowish watery diarrhea, and dehydration; mortality was 20–30% (20). Diarrhea, lethargy, and loss of appetite were associated with rotavirus infection in 3–4-month-old racing pigeons in the United Kingdom (26).

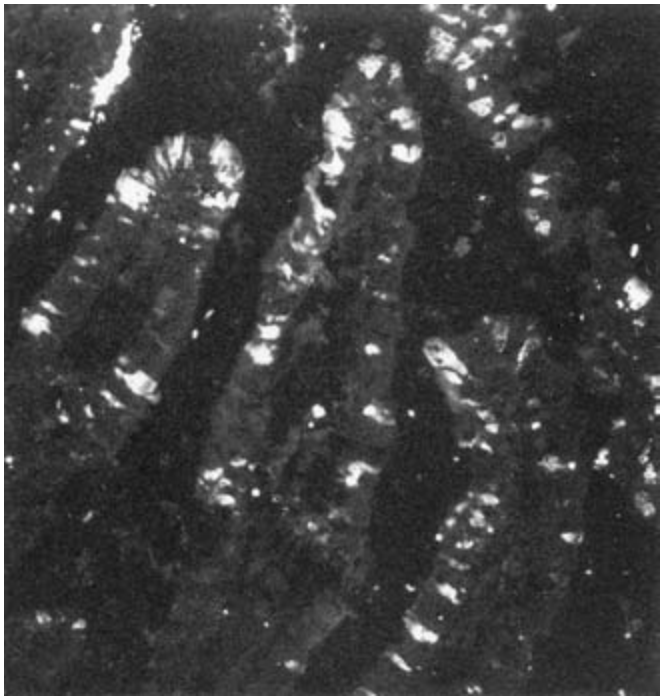
Variations in severity of clinical signs associated with rotavirus infections could be due to genuine differences in virulence of avian rotavirus strains, as has been shown for mammalian rotaviruses (19, 50), and interaction of rotavirus with additional factors such as other infectious agents (33) or environmental stress.

Morbidity is high. Most fecal specimens taken randomly from birds in affected flocks will contain rotaviruses.

Pathology

Gross

The most common finding at necropsy is the presence of abnormal amounts of fluid and gas in the intestinal tract and ceca. Pallor of the intestinal tract accompanied by loss of tonicity may be evident. Secondary findings may include dehydration, stunting of growth, pasted vents, inflamed vents, anemia due to vent pecking, litter in the gizzard, and inflammation and encrustation with droppings of plantar surfaces of the feet (7, 33, 34, 37, 57, 62, 98, 122). Hemorrhages were observed in the cecal walls of some experimentally infected pheasant chicks (28), and discrete, multifocal, superficial brownish-red erosions were found in the duodenum and jejunum of turkeys experimentally infected at 84 and 112 days of age (122).

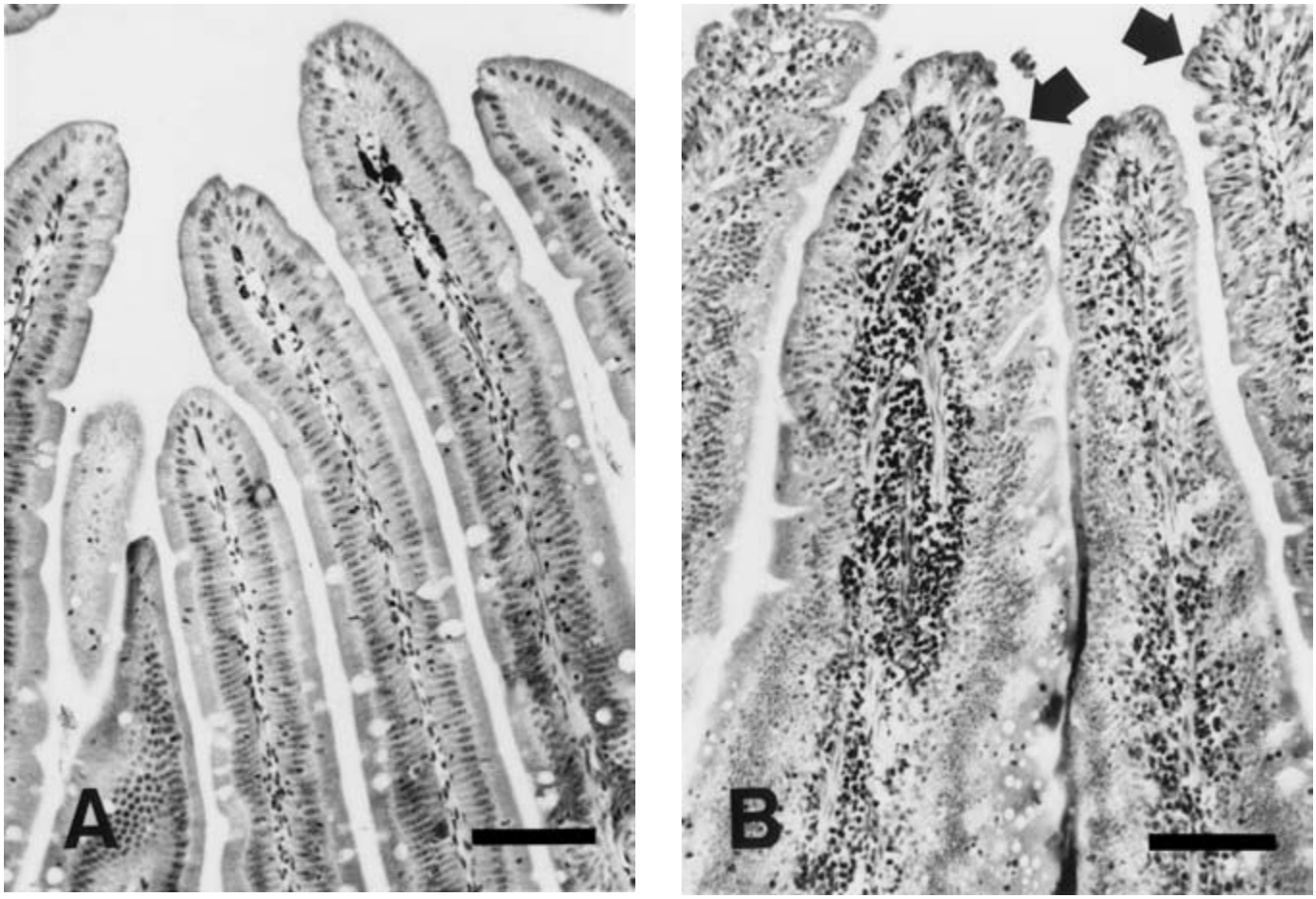


12.8. Immunofluorescent staining of duodenum of specific-pathogen-free chicken infected with rotavirus at 14 days of age and killed 3 days postinfection; rotavirus antigen is seen in villous epithelial cells. ×96. (*Avian Pathology*)

Microscopic

Immunofluorescence (IF) studies using chickens and turkeys experimentally infected with rotavirus have demonstrated the principal site of virus replication to be the cytoplasm of mature villous absorptive epithelial cells in the small intestine. Infected cells were most numerous in the distal third of villi (Fig. 12.8). Small numbers of infected cells were also detected in colon epithelium, ceca, cecal tonsils, and lamina propria of some villi. No IF was observed in proventriculus, gizzard, spleen, liver, or kidney (57, 67, 119, 120, 121). Within the small intestine, different rotavirus strains may show preference for specific areas. A group A rotavirus grew best in the duodenum of experimentally infected chickens, and a group D rotavirus favored the jejunum and ileum (57). In general, experimental infections using chickens and turkeys of differing ages showed that increasing amounts of viral antigen were detected in birds of increasing age (121).

Microscopic lesions in the small intestines of turkeys experimentally infected with group A rotaviruses consisted of basal vacuolation of enterocytes, separation of enterocytes from the lamina propria with subsequent desquamation, villous atrophy accompanied by widening of the lamina propria, scalloping of the villus surface, fusion of villi, and leukocytic infiltration of the lamina propria (98, 122). In general, mean villous lengths were decreased and crypt depths were increased following experimen-



12.9. Duodenum of SPF turkey poults. A. Normal villi of an uninfected control poult at 10 days of age. B. Villi of a 10-day-old poult infected with Tu-2 rotavirus at 7 days of age. Note the remarkable hypercellularity in the lamina propria, scalloping of the villous surface, and basal vacuolation of the epithelial cells at the tips (arrows). H&E stain, Bar = 0.1 mm. (120)

tal infection, resulting in decreased villus to crypt ratios; morphometric changes were more pronounced in the duodenum and jejunum than in the ileum (33, 98, 122). There was infiltration of polymorphonuclear and mononuclear cells into the lamina propria of the cecum and colon in some birds (122). Scanning electron microscopy demonstrated roughened villus surfaces, irregularly shaped and sized villi (33, 122), and loss of microvilli in enterocytes located at the tips of villi (122). In experimentally infected chickens, only minimal leukocytic infiltration of the lamina propria, with minimal loss of microvilli on cells at villus tips, was found in one study (122). Moderate villus atrophy, mainly in the ileum, however, has also been described by other workers in experimentally infected chickens (67). Microscopic changes observed in experimentally infected SPF turkeys are shown in Figure 12.9.

Similar lesions to those found in turkeys were reported in pheasant chicks experimentally infected with a group D rotavirus. However, when microscopic lesions were most severe, at 7 days PI, viral antigen was detected along the entire length of villi and occasionally in the crypts. Lesions were most severe in the duodenum and jejunum (34).

No histopathologic changes were observed in poults with naturally acquired rotavirus infection (37). Degeneration and inflammation of villi of the duodenum and jejunum have also been reported, however, in poults with rotaviral enteritis (7). Lesions were not found in ileum, cecum, colon, cloaca, or other organs.

Neither gross nor microscopic lesions are pathognomonic for rotavirus infection.

Ultrastructural

No detailed, sequential studies of the ultrastructural pathology associated with avian rotavirus infections have been published. The major ultrastructural changes are illustrated in Figure 12.6.

Pathogenesis of the Infectious Process

Little information about the pathogenesis of avian rotavirus infections exists, but inferences can be drawn from what is known about mammalian rotaviruses. With both avian and mammalian rotaviruses, the target cells are mature columnar absorptive cells that are located in the villous epithelium. Studies with whole virus and with purified VP8, the cell attachment protein, indicate that when initiating infection, avian rotaviruses utilize sialic acid-

containing molecules as receptors on the surface of MA104 cells (99). It is not known if a similar mechanism operates *in vivo.*

Under normal circumstances, villous epithelial cells have a relatively short life in mammals and birds, and they desquamate from the tips of the villi. They are replaced by a process of cell division in the crypts, followed by migration of cells up the sides of the villi to the tips. Crypt cells are immature and poorly differentiated. As they migrate up the villi, they differentiate and start to produce disaccharidases, alkaline phosphatase, and mechanisms to transport sodium. In rotavirus-infected piglets, infection and destruction of mature villous epithelial cells results in increased division and accelerated migration of crypt cells, so that the villi are clothed with immature, poorly differentiated cells, deficient in disaccharidases, alkaline phosphatase, and (Na^+-K^+)-ATPase. Glucose-stimulated sodium transport and the net absorption of sodium, potassium, chloride, and water are also decreased, producing a rapid-onset, severe, watery diarrhea with loss of electrolytes in the feces (31).

It is assumed that broadly similar mechanisms operate in rotavirus-infected birds. Decreased absorption of D-xylose from the intestinal tract has been observed in experimentally infected turkeys (33, 98, 121). However, shortening of the villi in experimentally infected turkeys (33, 98, 122) and chickens (122) was less severe than in infected calves and piglets. It has been suggested that the differences in lesions between infected birds and mammals and the age-related susceptibility of turkeys and chickens may result from differences in the development of intestinal villi between mammals and birds (120, 121, 122). It has been postulated that the frothy fluids found in the ceca of infected birds may result from impaired digestion and absorption of carbohydrates and sugars that, in turn, lead to their fermentation by cecal bacteria, producing metabolites that draw water into the ceca by osmosis (120).

However, malabsorption may not be the only explanation for rotavirus-induced diarrhea. NSP4 proteins of mammalian rotaviruses have been shown to be enterotoxins, causing diarrhea in suckling mice (5, 50). Avian rotavirus NSP4 glycoproteins have been shown to have similar biological activity, despite major differences in the amino acid sequences of avian and mammalian rotavirus NSP4 proteins (72, 73). It is believed that mammalian rotavirus NSP4 activates a signal transduction pathway that increases intracellular calcium levels in cells by mobilizing calcium from the endoplasmic reticulum, resulting in an increase in plasma membrane chloride permeability and potentiating chloride secretion, thereby producing a secretory diarrhea (5, 50).

There are many reports of detection of a rotavirus viremia and of extraintestinal infection/presence of rotaviral antigen in a wide variety of naturally and experimentally infected mammals. It has recently been shown that in experimentally infected rats, rotavirus infects and causes histopathological changes in cells in the liver and lungs, and replicates in macrophages in blood vessels and in the lungs, providing a possible mechanism for widespread dissemination through the body (14). However, it is not known if a similar situation occurs in infected birds.

Immunity

Active

Chickens and turkeys inoculated orally with rotaviruses showed serum antibody responses as early as 4–6 days postinfection measured by indirect IF. In general, older birds developed higher antibody titers and responded more quickly than younger birds (119, 120, 121). Little is known about development or duration of immunity to rotaviruses following infection of birds. Using immunoglobulin class-specific enzyme-linked immunosorbent assays (ELISAs) (78) to follow antibody responses in chickens experimentally infected with a group A rotavirus, rotavirus-specific IgM, IgG, and IgA were detected in serum; whereas the intestinal antibody response consisted almost entirely of IgA. Embryonic bursectomized chicks recovered from infection and developed resistance to a subsequent homotypic challenge more slowly than intact chicks (76), indicating that the intestinal IgA response is not the sole mediator of recovery from infection and development of resistance to reinfection, but that it plays a part. Natural killer cell-like activity has been demonstrated in chick intraepithelial leukocytes against rotavirus-infected target cells, and this may be an important *in vivo* immune response (77).

Passive

Maternally derived antibodies to rotavirus are passively transferred to the avian embryo through the egg yolk. They progressively decline in titer in the serum and are undetectable at 3–4 weeks of age (56, 120). Presence of maternal antibody in the serum had no apparent effect on susceptibility of chickens and turkeys to experimental group A rotavirus infection (67, 120). Progeny of hyperimmunized turkey hens, however, were more resistant to experimental infection with rotavirus at 2 or 5 days of age, but not at 12 days of age, compared with poults without circulating maternal IgG antibodies to rotavirus. It was suggested that circulating maternally derived IgG protects the intestinal mucosa against rotavirus infection during the first week of life and that this effect depends on the titer of antibodies (67, 98, 120). It was subsequently shown that, during the first week of life, maternally derived anti-rotavirus IgG (rIgG) titers in intestinal washings of poults derived from hyperimmunized (vaccinated) hens were 200–500-fold less than rIgG titers in serum and that intestinal titers at 10 and 13 days of age were negligible. Evidence suggested that the rIgG had been transferred from the blood to the intestine. However, maternally derived rIgG could not be detected in intestinal washings of progeny derived from naturally infected hens (97).

Similarly, an increase in serum neutralizing antibodies was observed in pheasant hens vaccinated with an inactivated group A pheasant rotavirus vaccine, compared with mock-vaccinated controls. When challenged with a group A rotavirus at 1–2 days of age, mortality in the progeny of the vaccinated hens was 19.4% and mortality in the progeny of the controls was 48.3% (25). These results and those cited previously for the progeny of vaccinated turkeys suggest (i) that maternally derived antibodies in the progeny of unvaccinated turkeys and pheasants are unlikely to provide significant protection against a field challenge with rotavirus; and (ii) that much higher titers of antibody would need

to be produced by vaccination to completely protect young birds even for the first week of life.

Diagnosis

Isolation and Identification of Causative Agent

The classic way to diagnose avian rotavirus infections in the laboratory is to identify the virus in feces or intestinal contents by direct electron microscopy. This technique is relatively sensitive and detects rotaviruses of all serogroups. Material can be prepared in a variety of ways (63). The standard method is to extract an approximately 15% suspension of feces made in phosphate-buffered saline with an equal volume of fluorocarbon. Following centrifugation at 3000 × *g* for 15–30 minutes to separate aqueous and fluorocarbon phases, the aqueous phase is removed and centrifuged at approximately 12,000 × *g* for 15 minutes using an Eppendorf 5414 bench centrifuge. This pelleting procedure gives similar results to those obtained by ultracentrifugation, but is quicker and simpler. The pellet is resuspended in a few drops of water and examined. Some workers use immune electron microscopy. Although this technique requires availability of specific antisera, it allows rotaviruses of different serogroups to be distinguished (93, 105). The morphology of rotavirus is sufficiently distinct that experienced electron microscopists should have little difficulty identifying the virus with certainty. Rotaviruses can be confused with reoviruses, however, which are also frequently found in avian feces. The main distinguishing feature is the more clearly defined outer capsid shell of rotavirus (see Fig. 12.5).

Detection of rotavirus RNA in intestinal contents or feces provides an alternate means of diagnosis. Following extraction of RNA, electrophoresis on polyacrylamide gels, and silver staining, rotavirus RNA can be identified by the pattern of migration of the 11 genome segments. This technique is almost as sensitive as electron microscopic techniques (54, 64, 103, 105), provides provisional information on the serogroup(s) present, and is a convenient means of distinguishing between different isolates. At present, this technique is used mostly by those interested in rotavirus epidemiology and classification.

Using feces samples collected from turkeys experimentally infected with a group A rotavirus, it was found that negative contrast electron microscopy was more sensitive than a staplylococcal protein-A coagglutination test and a commercial ELISA developed for mammalian group A rotavirus detection; the latter two tests had approximately the same sensitivity (87% and 90%, respectively) and were slightly more sensitive than virus isolation in MA104 cells (44). Commercially available ELISAs are commonly used to detect group A rotaviruses in mammalian and avian feces. However no available ELISAs detect rotaviruses of groups D, F, and G. Given the frequent occurrence of infection with these serogroups in poultry, use of commercial ELISAs for diagnosis in poultry will result in many infections being missed.

Diagnosis of rotavirus infection by virus isolation in cell cultures is useful only for group A avian rotaviruses. It has proven extremely difficult to isolate other rotavirus serogroups in cell cultures (16, 46, 64, 107). As infections with other serogroups constitute the majority of rotavirus infections in chickens (64, 109) and turkeys (86, 108), virus isolation in cell cultures cannot be recommended as a diagnostic technique. Even with group A avian rotaviruses, in most cases, serial passage can be achieved only by activation of virus infectivity with proteolytic enzymes such as trypsin. Furthermore, not all group A avian rotaviruses detected by electron microscopy grow in cell cultures. Those that do are often noncytopathic on primary isolation, requiring immunofluorescence to detect virus growth. For isolation of group A avian rotaviruses, the MA104 cell line or primary cultures of chick embryo liver or chick kidney cells, trypsin treatment, and centrifugation of inoculum onto the cell monolayer are recommended (46, 60, 66, 69, 75, 100, 107, 118).

It is anticipated that polymerase chain reaction methods developed to detect rotavirus nucleic acid in the feces of mammalian species (29, 116, 117) will be used for diagnosis in avian species.

Serology

Serologic diagnosis of rotavirus infections is difficult and not recommended. The high prevalence of antibody (58, 69) makes results difficult to interpret. Few laboratories offer serologic tests for avian rotaviruses on a routine basis. Furthermore, the inability to adapt some avian serogroups to cell culture has resulted in gaps in the available battery of antigens. Serologic screening using indirect immunofluorescence (58) or ELISA (78) is useful for establishing and monitoring the status of specific-pathogen-free flocks.

Differential Diagnosis

Rotavirus infection must be differentiated from other conditions causing diarrhea. Because the clinical signs and pathology of rotavirus infection are not pathognomonic, laboratory diagnosis is necessary. It is important to remember, however, that rotavirus infection does not necessarily result in disease, as discussed previously. Furthermore, it is not unusual to find other potential viral enteropathogens, in addition to rotavirus, in flocks with enteric disease (3, 23, 35, 80, 86, 93, 123). Thus, diagnosis of outbreaks of enteric disease can be problematical.

Intervention Strategies

Management Procedures

The ubiquity of rotavirus infections in turkeys and chickens indicates that it is not practical to keep commercial flocks free from infection. At present, no specific treatment or means of control exists. The effect of diarrhea on the litter can be minimized by increasing ventilation rate and temperature and by adding fresh litter. Where litter is reused several times, infection will build up, and problems are likely to be more severe than in situations in which houses are cleaned and fumigated and fresh litter is used for each batch of birds. If severe problems arise, remove litter and thoroughly clean the house and equipment and fumigate with formaldehyde before restocking with a new flock.

Vaccination

Commercially available vaccines have not yet been developed. Given the extent of antigenic diversity that exists in avian ro-

taviruses and the difficulty in growing nongroup A rotaviruses in cell culture, obvious problems exist in vaccine development. Preliminary work on experimental group A rotavirus vaccines in turkeys (97) and pheasants (25) indicates that inactivated vaccines administered to the breeders are unlikely to protect the progeny against challenge for more than the first week of life, unless much higher titers of anti-rotavirus antibody can be produced.

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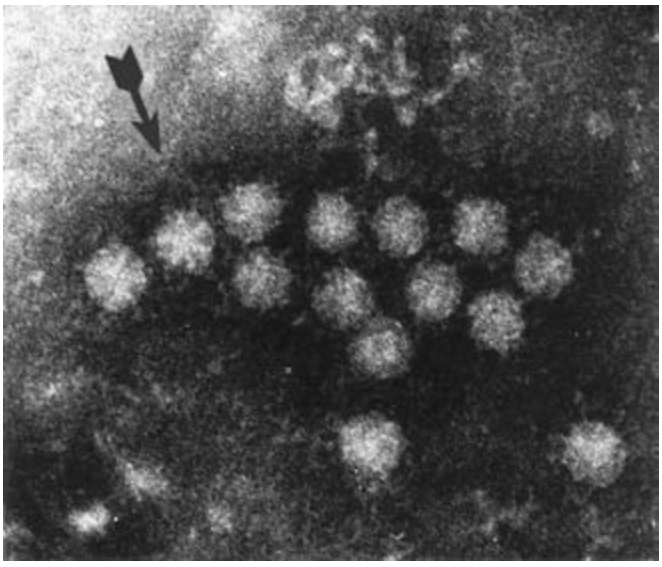
Astrovirus Infections

D. L. Reynolds and S. L. Schultz-Cherry

Introduction

Astroviruses cause, or have been associated with, acute gastroenteritis in humans, cattle, swine, sheep, cats, dogs, deer, mice, turkeys, guinea fowl as well as fatal hepatitis in ducks (1, 4–6, 9, 15, 22, 23, 28, 29, 34, 35, 41, 59, 52–54). Chickens can be infected with astroviruses that are genetically more similar to turkey astrovirus strains (2) or with avian nephritis virus (ANV) (10). However, ANV will not be covered in this chapter (see Chapter 14). More recently, astroviruses have been detected in birds with poult enteritis and mortality syndrome (PEMS); although the exact role of astroviruses in PEMS remains unclear (10, 20, 21, 55, 56). The economic impact of astrovirus infections on the poultry industry has yet to be determined. Whether or not avian astroviruses can infect other animal species, including humans, constituting a public health concern, is also unknown. Intriguingly, recent studies demonstrated that antibodies to chicken astrovirus (CAstV) were found retrospectively in some turkey flocks tested in 1982 suggesting that cross-species infection may occur (2).

Astroviruses were first identified in 1980 by McNulty *et al.* (28) from the intestinal contents of 11-day-old turkey poults with diarrhea and increased mortality. Subsequently, astroviruses in flocks of young turkeys have been reported in the United States since at least 1985 (7, 8, 19–21, 31, 34, 35, 38, 40, 55, 56).



12.10. A star-shaped astrovirus particle (arrow) among an aggregate of astroviruses from intestinal samples of experimentally infected diarrheic poults, detected by immune electron microscopy. Average particle size is 29.6 nm. (*Avian Diseases*)

Incidence and Distribution

Astrovirus infections are geographically widespread and are usually the most prevalent virus infection, other than rotavirus infection, in poults 1–5 weeks old with enteric disease (34–36). In one study, astrovirus infections occurred in nearly 80% of affected flocks and was the most prevalent virus detected (36). Astroviruses have also been detected in normal healthy flocks but far less frequently (30%). Astroviruses are seldom the only virus detected in flocks with enteric disease. Generally, they occur with other enteric viruses, especially group D rotavirus (36).

Astrovirus infections typically occur within the first 4 weeks of life (35). When flocks were continuously monitored for enteric viral infections from 1 day of age until market, the first samples positive for viruses always contained astroviruses, either alone or with other viruses (35).

Etiology

Astroviruses are small, round viruses typically 25–35 nm in diameter typically spread via the fecal-oral route. They get their name from the 5 or 6 pointed star-like surface projections observed using electron microscopy (EM); see Figure 12.10. However, only 10% of astroviruses in a population may exhibit this morphology, and visualization is dependent on sample preparation (19, 21, 23, 25–27, 29, 52).

Astroviruses are nonenveloped, positive sense RNA viruses. Their viral genome is 6.5–7.5 kilobases (kb) long and contains 3 open reading frames (ORF). These reading frames code for nonstructural proteins (ORF1a), a viral RNA-dependant RNA polymerase (ORF1b), and precursor capsid protein (ORF2).

Astroviruses are distinct molecularly from picornaviruses in that they synthesize a subgenomic message during replication and differ from picornaviruses and caliciviruses in that they have a retrovirus-like frame shift signal sequence between ORF1a and ORF1b (19, 21, 27). Currently, only the human astrovirus (HAstV), 2 distinct strains of turkey astrovirus (TAstV), swine astrovirus (SAstV), and avian nephritis virus (ANV) have had their genomes fully sequenced (10, 12–14, 21, 50, 51). The avian astroviruses are molecularly distinct, sharing very little sequence similarity in the different gene segments. However, the turkey astroviruses cluster into a distinct group from ANV.

Similar to the human astroviruses, there is antigenic variation among the TAstVs (19). The prototype TAstV-1 strain was originally identified in the United States in 1985 and is antigenically and serotypically distinct from North Carolina/96, the prototype TAstV-2 isolate. It is evident that even within a single TAstV genotype, there are distinct subtypes. Tang *et al.* demonstrated that the astrovirus isolates TAstV1987 and TAstV2001 are antigenically and serotpically distinct with TAstV1987 being more closely related genetically to the prototype NC/96 (44, 45).

Further, Guy *et al.* demonstrated that a "small round virus" originally identified as an enterovirus is related to TAstV-2 (8). A recent study evaluated the genetic diversity of TAstV-2 isolates collected from across the United States and performed comparative analysis of the polymerase and the capsid genes. These studies clearly demonstrated that even within the more conserved polymerase gene, there is at least 10% nucleotide divergence supporting the assortment of isolates into 2 distinct groups (31). The nucleotide and amino acid sequence variation within the capsid region revealed substantial variation among the isolates. Phylogenetically, the isolates assorted into 9 groups based on greater than 10% nucleotide sequence divergence within groups (31). Whether this sequence variation is simply due to the error-prone nature of the RNA-dependent RNA polymerase, evidence of immune pressure by the host, or is due to actual gene recombination as suggested in Pantin-Jackwood, Spackman *et al.* 2006 is a matter for continued debate.

Susceptibility to Chemical and Physical Agents

Mammalian astroviruses are extremely resistant to inactivation. Similarly, turkey astroviruses are also extremely stable. Astrovirus particles are resistant to inactivation by acidic pH, chloroform, a variety of detergents, heat, ambient temperatures, and lipid solvents (38). Additionally, Kurtz *et al.* (24) demonstrated that astroviruses are resistant to most alcohols, and only 90% methanol inactivates both purified astrovirus and astrovirus in feces.

Turkey astroviruses harvested from the intestines of embryos are very stable. Infectivity can be maintained for several weeks at 4°C. To maintain infectivity for the long term, virus can be stored at –20°C or –70°C. Even in the embryo model, astrovirus is resistant to inactivation with a variety of disinfectants including treatment with phenolics, acidic pH, chloroform, a variety of detergents, heat, ambient temperature, quarternary ammonia, and most alcohols (38). Formaldehyde, b-propriolactone, 90% methanol, and a peroxymonosulfate-containing disinfectant have been used to eliminate infectivity in the embryo model. Inactivation of astrovirus in the laboratory situation can be accomplished with 90% methanol or through the use of a peroxymonosulfate-containing disinfectant (38).

Laboratory Host Systems

Turkey astroviruses can be propagated serially in the yolk sac of 20-day-old turkey embryos or by inoculating 24–25-day-old turkey embryos by the amnionic route (3, 16–21). At 5 days postinoculation (dpi), the intestines and bursa can be isolated for virus purification by cesium chloride or for use as viral stock. In 75–80% of specific-pathogen-free (SPF) embryos inoculated with turkey astrovirus, the intestines are distended, thin-walled, and fluid-filled by 5 dpi. The astrovirus can be purified from the fluid as well as from the embryo intestines. This pathogenic effect is not observed in commercial embryos, although there is astrovirus replication. Astrovirus infection does not result in embryo mortality. To date, replication of turkey astrovirus in cell culture lines has been unsuccessful, even in the presence of exogenous trypsin.

In contrast, chicken astroviruses (CAstV) can be cultivated in cell culture (2). The CAstV isolates replicate well in primary chick embryo liver and LMH (chicken heptocellular carcinoma cell line) cells producing a marked cytopathic effect (CPE) after four-to-five passages. CAstVs replicate poorly in chick embryo fibroblasts and chick kidney cultures initially, but after several passages will replicate in chick kidney inducing CPE.

Pathogenesis and Epidemiology

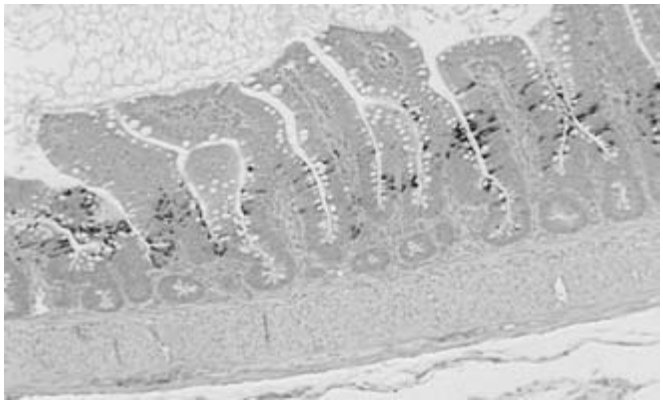
In commercial flocks, astroviruses have been isolated from turkey poults experiencing viral enteritis. Clinical signs of disease usually develop between 1 and 3 weeks of age and generally last 10–14 days (34). They vary somewhat, but typically include diarrhea, listlessness, litter eating, and nervousness. Severity ranges from mild to moderate with only slight mortality. Morbidity, occurring as decreased growth (stunting), is of greatest concern.

Experimentally, turkeys infected with TAstV-2 developed a profuse, watery diarrhea by 2 days post infection, which continued through 12 days post infection (18, 29, 34). Morphologically, intestines of infected poults were 3 to 5 times larger than their control counterparts and appeared dilated, distended, and fluid-filled by 3 days post infection (3). TAstV infection resulted in an overall growth depression of infected birds, possibly as a consequence of decreased absorption in infected birds, by 5 days post infection and throughout the experiment (18). When SPF poults were given an inoculum containing only astrovirus, they absorbed significantly less D-xylose compared with uninoculated control poults. Commercial poults inoculated with astrovirus had decreased intestinal maltase by 3 days postinfection. The decrease in specific maltase activity in astrovirus-infected poults was transient, returning to normal by 10 days postinfection (11, 48).

Histological and Gross Changes

At necropsy, characteristic pathologic changes were dilated ceca containing yellow, frothy contents, and gaseous fluid; loss of tone (gut thinness); and hyperemia of the intestinal tract. The pathogenesis of diarrhea associated with astrovirus infections has been at least partially attributed to the osmotic effect of undigested, unabsorbed disaccharides (and other nutrients) attracting water to the intestinal lumen.

Studies in the mid-1990s demonstrated that astrovirus infections of poults induced histopathologic lesions of the small intestine characterized by mild crypt hyperplasia, resulting in increased crypt depth and area (47). Histopathologic changes were noted in the proximal jejunum as early as 1 day postinfection, with all portions of the small intestines affected by 5 days postinfection. Unlike some other intestinal viral infections, astrovirus infections do not induce villous atrophy. In-situ hybridization studies with a probe specific to TAstV-2 demonstrated that replication was restricted to the intestines (see Fig. 12. 11).



12.11. *In situ* hybridization demonstrating the location of TAstV-2 replication in the intestine of poults 3 days post infection.

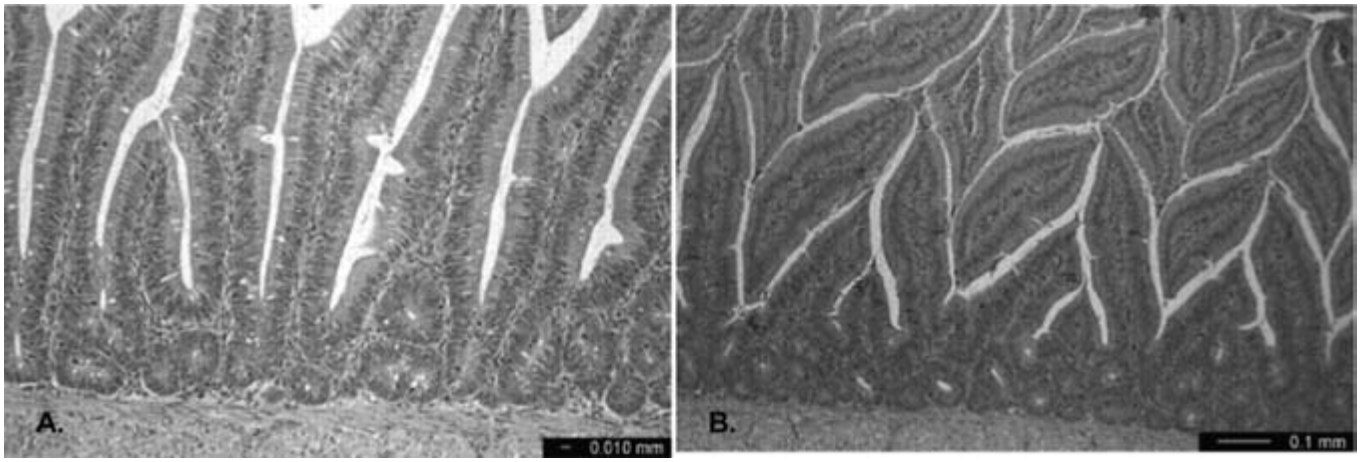
Replication was detected in the upper regions of the small intestine by 24 hpi (3). Individual degenerating enterocytes were apparent along the basal edge of villi by 2 dpi and continued through day four. By day five, mild shortening of the villi was observed (3) as well as occasional clusters of necrotic enterocytes along the villous base, correlating with infection (18). TAstV-2 was localized primarily to the large intestine by day 7 pi. Replication peaked at 3 to 5 days post infection and TAstV-2 was infrequently observed at time points later than 9 days post infection (3). Although replication was limited to the intestine, infections (18) TAstV-2 was isolated from the bursa, thymus, spleen, kidney, skeletal muscle, pancreas, and plasma (18). Overall, TAstV-2 infection, as shown in Figure 12.12, was associated with mild histopathology and little cell death as measured by TUNEL (18).

The lack of an inflammatory response may result from the increase in transforming growth factor–beta (TGF-ß), a potent suppressor of inflammation, in infected intestines. Intriguingly, the histopathology observed in the young turkey model is similar to that observed in human infection (39). These results suggest that astrovirus-induced diarrhea is due to a mechanism other than destruction of the intestinal epithelium or eliciting an inflammatory response and raise the question of how astroviruses induce diarrhea. Preliminary data suggests that astrovirus infection results in increased paracellular permeability and changes in ion regulation resulting in diarrhea (30).

Immune Response

Although viral replication was limited to the intestine, infectious TAstV-2 virus was isolated systemically and a transient viremia was observed 3 dpi (18). TAstV-2 infected turkeys also demonstrated a transient, localized growth depression of the thymus, which manifested by 5 days post infection and resolved by 12 days post infection (18). The effects of astrovirus on the immune response and the mechanism of viral clearance are under investigation (16).

Qureshi *et al.* demonstrated that the responsiveness of peripheral blood lymphocytes (PBLs) isolated from astrovirus-infected poults was significantly reduced as compared to control birds up until 7 dpi (33). Further, they reported that astrovirus infection caused a transient decline in circulating CD4- CD8+ lymphocyte populations (32, 33). This is in contrast to a separate study demonstrating that astrovirus infection had no effect on T-cell populations and that virus-specific antibodies were not substantially altered in response to TAstV-2 (17). Further, neutralizing antibodies to TAstV-2 have not been isolated from infected poults. To date, studies suggest that the innate immune response may be important for controlling primary astrovirus infection. During infection, macrophages are induced to produce nitric oxide (NO) in a replication-independent manner. In turn, NO inhibits viral replication both *in vitro* and *in vivo* suggesting an important role in control (17). An additional control mechanism appears to be the induction of type 1 interferon (IFN). Like many other viruses, type 1 IFN inhibits astrovirus replication *in vitro* and *in vivo* and astroviruses have evolved mechanisms to inhibit IFN production during infection to allow viral spread (30).



12.12. TAstV-2 infection results in minor histopathologic changes (B) as compared to mock-infected poults (A).

Diagnosis

Historically, immune electron microscopy was the primary method for identifying astroviruses from fecal and/or intestinal samples. This procedure is done by diluting the fecal/intestinal sample with sterile diluent such as phosphate-buffered saline (pH 7.2) to make a working solution. The diluted sample is thoroughly mixed by using a homogenizer or vortex mixer and sonicated. Particulate matter and bacteria are removed by centrifugation at 500 × *g* for 20 minutes and filtering the resulting supernatant fluid through a 450 nm porosity membrane filter. The filtrate is incubated with an appropriate dilution of antiserum containing astrovirus antibodies. Following incubation, the sample is pelleted by ultracentrifugation, negatively stained with phosphotungstic acid, and observed by EM. Aggregates of astrovirus can be observed easily at 330,000–50,000× magnification. Although astroviruses have star-shaped morphology, only a small percentage of particles display this characteristic. It is quite difficult to diagnose astrovirus infections accurately without IEM. A definitive diagnosis of astrovirus infection is made by recognizing aggregates of typical astrovirus particles in the IEM preparation. In the author's experience, turkey astroviruses only occasionally display nonspecific agglutination; therefore, one must rely on IEM for aggregation of particles (Fig. 12.10).

Recently, several diagnostic tests have been developed that specifically detect the distinct TAstV strains. Two antigen-capture enzyme-linked immunosorbent assays (AC-ELISAs) were developed that could detect TAstV-1 and TAstV-2 strains in intestinal homogenates. The basis of the assay is a capture antibody, which is coated on a 96-well plate, intestinal homogenates from suspect flocks are added, and the presence of TAstV antigen is detected with a second specific antibody. The assay was unable to detect virus in fecal or non-intestinal tissue homogenates, but detected virus in intestinal homogenates with a titer of 5×10^2 egg infectious dose 50 (43). Because there is no "gold standard" test for TAstVs, the authors were unable to test the specificity and sensitivity of the AC-ELISA.

Several reverse-transcriptase polymerase chain reaction (RT-PCR) tests have been developed to identify astrovirus in feces or intestinal homogenates (20, 43). This procedure is completed by pooling the feces or lower intestines from 3–5 birds per flock and either isolating RNA directly or passaging filtered fluids one time through embryonated turkey eggs. The RNA from the field sample or from the isolated embryo intestines then is subjected to RT-PCR using oligonucleotide primers specific to 2 different genes of the viral genome, a conserved region and a diverse region. The use of primers from 2 different regions of the virus allows the identification of distinct astrovirus genotypes. Through the development of this test, it is possible to distinguish the astrovirus that circulated among commercial turkey flocks in the 1980s from the more recent NC96 strain. The RT-PCR test will also detect the presence of astrovirus as early as 1 day postinfection. The sensitivity, specificity, and cost of the RT-PCR test has been greatly improved by the development of several real-time RT-PCR assays (RRT-PCR) (17, 42). RRT-PCR amplifies viral nucleic acid similar to standard RT-PCR, but the product is detected in "real time" with a sequence-specific probe labeled with fluorescent dyes. RRT-PCR is commonly used in human diagnostic laboratories and now serves as the "gold standard" test for numerous viral pathogens.

Differential diagnosis of astrovirus infections in turkey poults needs to include infectious, parasitic, and noninfectious agents that can cause enteric disease. Cultures for enteropathogenic bacteria, such as *Salmonella* spp. and *Campylobacter* spp., should be done. Smears or tissue sections should demonstrate protozoa. Other enteric viruses need to be excluded including coronaviruses, reoviruses, rotaviruses, and toroviruses. Differential diagnosis of several viral enteric pathogens can now be accomplished by multiplex RT-PCR or RRT-PCR (40, 42, 46). These assays differentiate turkey astrovirus, turkey coronavirus, and turkey reovirus in intestinal homogenates. The rapid advances in diagnostic technology make it probable that we may one day have an affordable enteric pathogen chip capable of rapidly identifying the causative agent(s) of infectious enteritis in poultry.

Treatment, Prevention, and Control

No vaccines, chemotherapeutics, or other measures are reported to be efficacious for control and/or prevention of astrovirus infections. Generally, good management practices emphasizing cleaning, disinfecting, litter management, and resting of facilities between flocks are recommended. Astrovirus infections have, however, continued to be problematic for some producers with modern facilities using high standards of management, suggesting that contemporary management practices may not have been entirely effective.

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Avian Enterovirus-Like Viruses

J. S. Guy, M. S. McNulty and C. S. Hayhow

Introduction

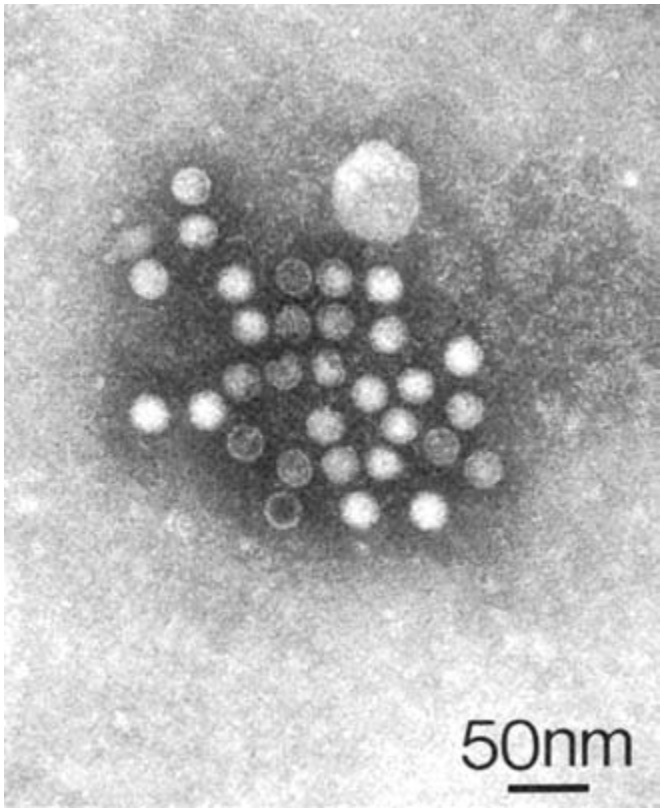
A number of enterovirus-like viruses (ELVs) have been identified in avian species. The term enterovirus-like is applied to these viruses, as they have not been fully characterized; definitive classification awaits further biologic, physicochemical, and molecular characterization. This section addresses those ELVs identified in domestic poultry, other than duck hepatitis virus types 1 and 3 (see Chapter 13), and turkey viral hepatitis virus (see Chapter 14).

The economic significance of avian ELVs is not yet known. No evidence suggests that they are transmissible from avian species to humans or other mammals. The extent, if any, to which they spread among different species of domesticated poultry is unknown.

Etiology

Classification

Enteroviruses comprise one of nine genera within the family Picornaviridae (40). Members of the Picornaviridae contain a single molecule of infectious, positive sense, single-stranded RNA, 7–8.8 kb in size. Genera within the family Picornaviridae are distinguished *inter alia* by their sensitivity to acid, buoyant density of the virion in CsCl, and clinical manifestations in the affected host. Members of the genus *Enterovirus* are stable at acid pH, have a density of 1.30–1.34 g/mL in CsCl, and they replicate primarily in the intestinal tract (21, 40). Most avian ELVs have been classified on the basis of size, morphology, cytoplasmic replication in enterocytes, and resistance to acid pH. However, it is emphasized that these biological criteria are insufficient for definitive classification. This is borne out by nucleotide sequence analyses of genomic RNA, and antigenic analyses, of some avian viruses that possess these biological criteria. Avian encephalomyelitis virus, a virus that initially was considered to be an enterovirus, has been shown to share a high level of deduced amino acid sequence identity with hepatitis A virus (24, 43). Based on these findings, avian encephalomyelitis virus has been provisionally classified as a tentative species in the genus *Hepatovirus* in the family Picornaviridae (40). Similarly, nucleic acid sequence analyses of other viruses—avian nephritis virus and two viruses initially thought to be turkey ELVs—have identified these viruses as members of the family Astroviridae (14, 15, 20, 22, 32, 40, 45, 46). Based on antigenic analyses, it is likely that several viruses initially identified as chicken ELVs will be reclassified in the future as astroviruses, as these viruses have been shown to share antigenic relationships with avian nephritis virus (4, 10, 29, 42). Furthermore, given the extent of antigenic variation exhibited by astroviruses, some of the ELVs that are antigenically unrelated to avian nephritis virus may also be reclassified at a future date.



12.13. Spherical, 18–27 nm enteroviruslike viruses (ELVs) detected in feces of young turkeys with enteric disease, sodium phosphotungstate.

Morphology

Picornavirus virions are icosahedral (T = 1), nonenveloped, and 22–30 nm in diameter. The virion lacks obvious surface structure, and no surface projections exist (21, 40) (Fig. 12.13). The sizes described for most avian ELVs fall within a 22–30 nm range, although a range of 18–24 nm was described for a U.S. turkey ELV (41).

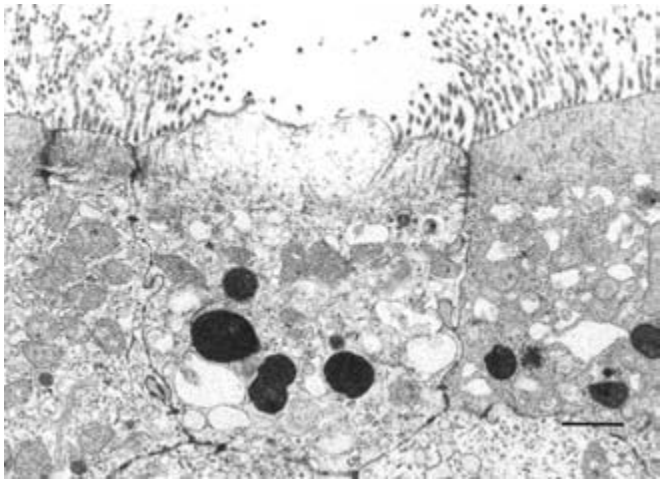
A turkey ELV isolate was determined to have a buoyant density of 1.33 g/mL in CsCl (18).

Chemical Composition

Only limited information is available regarding the chemical composition of avian ELVs. Information on the genome structure of ELVs is available only for a single U.S. isolate from turkeys (18). This virus was shown to possess a single-stranded RNA genome of approximately 7.5 kb. No information is available regarding avian ELV proteins.

Virus Replication

Replication of turkey ELVs has been investigated by both immunohistochemistry and thin-section EM (19). Virus replication



12.14. Degenerating enterocyte containing cytoplasmic crystalline arrays of enteroviruslike viruses (ELVs).

was shown to occur in the cytoplasm of intestinal enterocytes. Crystalline arrays composed of small, round virus-like particles approximately 23 nm in diameter (Fig. 12.14) were observed (19); an earlier study (41) described particles of 17.1 nm. Similar findings to the former were reported for chicken ELVs (6, 9, 25, 27). However, in one detailed study (9), membrane-bound cytoplasmic inclusions containing virus-like particles were detected more frequently in mesenchymal cells and macrophages in the lamina propria than in enterocytes. Some chicken ELVs also replicate in the kidney, and have been implicated in the etiology of baby chick nephropathy (37).

A U.S. turkey ELV was shown by immunofluorescence and immunoperoxidase staining procedures to replicate primarily in the jejunum and ileum of experimentally infected poults. The virus replicated preferentially in those enterocytes located halfway between the tip and base of the villus. Viral antigen was found most abundantly in enterocytes situated immediately above crypt openings (17); similarly, antigens of chicken ELVs were found mostly in cells at the base of the villi (6).

No information is available concerning transcription and translation of the RNA of avian ELVs.

Susceptibility to Chemical and Physical Agents

Avian ELVs that have been tested have been found to be stable at pH 3 and unaffected by solvents, such as chloroform and ether (25, 27, 28, 39, 42). No information exists about their sensitivity to disinfectants.

Strain Classification

Because of the difficulties associated with growing avian ELVs in cell culture and other laboratory host systems, very little information is available concerning their antigenic relationships. Using cross immunofluorescence, 3 ELVs isolated from chickens, designated EF84/700 (28), FP3 (39), and 612 (25), were found to be antigenically distinct from each other and also from avian encephalomyelitis virus, avian nephritis virus, duck hepatitis virus type 1 and duck hepatitis virus type 3 (25, 29). Several ELVs isolated in Japan from chicks with baby chick nephropathy (37) and from broilers with a stunting syndrome (42) had biologic and physical properties similar to the G-4260 strain of avian nephritis virus but were antigenically distinct from avian nephritis virus (36,37). Nucleotide sequence analyses of the genomes of these viruses is needed to determine whether these viruses are enteroviruses or a third serotype of avian nephritis virus (36, 37).

Two strains of turkey ELVs isolated in France were shown to be antigenically unrelated to avian encephalomyelitis and duck hepatitis viruses using cross-neutralization tests (1).

Laboratory Host Systems

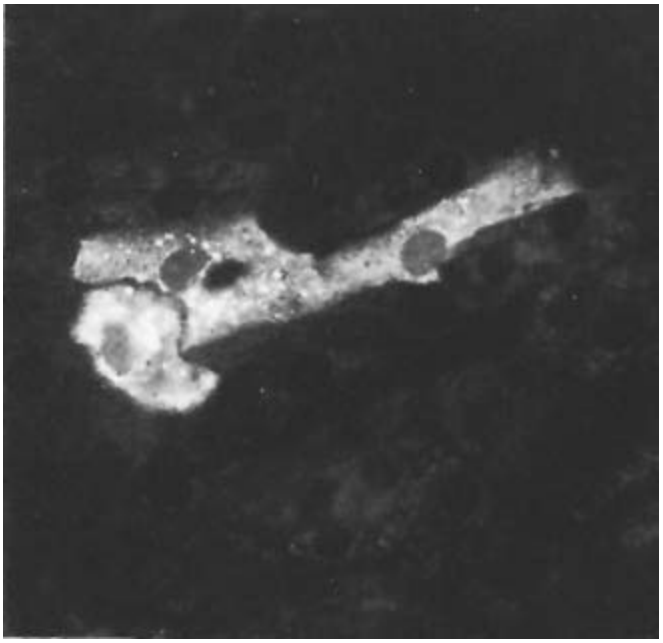
Enterovirus-like viruses can be propagated in the laboratory by oral inoculation of neonatal birds of the same species from which they originally were recognized or isolated. Depending on the virus, inoculated birds may develop enteric disease and depressed growth rates. Intestinal contents examined by negative contrast EM 1–3 days postinfection (PI) normally will contain the inoculated virus. Additionally, immune electron microscopy can be used to assist identification of ELVs in intestinal contents of inoculated birds (33, 34, 41). However, caution must be exercised in propagating ELVs in this manner as even specific-pathogen-free birds may be infected with ELVs.

Most chicken ELVs will grow in the yolk sac of 6-day-old embryonated chicken eggs, with approximately 50% of embryos dying within 3–7 days PI. Dwarfing of embryos may also be observed (37). Some of these viruses also can be propagated in the chorioallantoic membrane of embryonated eggs. Immunofluorescent staining of impression smears of yolk sac membranes or cryostat sections of chorioallantoic membrane can be used to confirm virus growth. In addition, some ELVs, for example FP3 and 612, show limited growth in primary cultures of chicken embryo liver or chicken kidney cells. Growth of virus in cell cultures is best detected by immunofluorescent staining (Fig. 12.15), as many of these viruses cause little, if any, cytopathology (4, 25, 29).

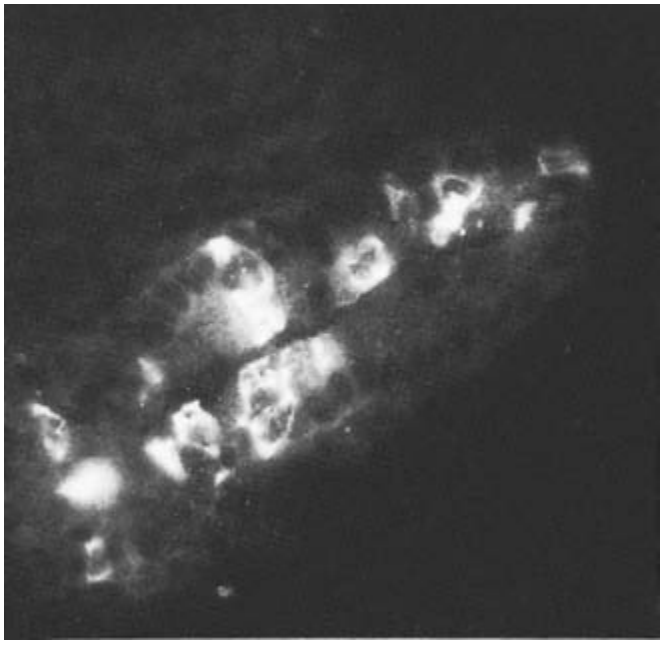
Samples of feces or intestinal contents that contain chicken or turkey ELVs also may contain reovirus. Reoviruses normally outgrow ELVs in embryos and cell cultures; thus, their presence interferes with attempts to isolate ELVs.

None of 4 turkey ELVs and 2 pheasant ELVs detected by negative contrast EM in the feces and intestinal contents of birds with enteric disease in the United Kingdom produced a cytopathic effect in primary chicken embryo liver cell cultures. However, 1 turkey virus grew to low titers in embryonated chicken eggs inoculated via the yolk sac (12). Similarly, 2 isolates of ELVs were made in France from turkey intestinal contents following yolk sac inoculation of chicken embryos (1).

An ELV from guinea fowl with transmissible enteritis was propagated successfully following inoculation of 7-day-old guinea fowl embryos via the yolk sac; however, embryo mortality and lesions were inconsistent. This virus also grew in primary cultures of guinea fowl embryo brain cells; no cytopathic effect was evident, but the presence of virus was demonstrated by inoculation of guinea fowl embryos and 1-day-old guinea fowl (31).



12.15. Immunofluorescent staining of chicken embryo liver cell culture infected with enteroviruslike virus (ELV) (612 isolate). ×450.



12.16. Specific immunofluorescence in epithelium of jejunal villus of chicken infected with enteroviruslike virus (ELV) (612 isolate). ×450.

Pathogenicity

The pathogenic role of avian ELVs requires further clarification. Although field and experimental evidence suggests that they may cause enteric disease in young turkeys, chickens, and guinea fowl, and nephropathy in baby chicks, additional studies are needed to define their importance.

Pathobiology and Epidemiology

Incidence and Distribution

Examination of feces using negative contrast electron microscopy (EM) has led to the discovery of ELVs in a number of avian species. The presence of ELVs in intestinal contents of young turkeys and chickens was described in the United Kingdom in 1979 (26). Subsequently, ELVs were identified in the feces of turkey poults in the United States (33, 34, 35), Italy (31), and France (1); in chickens in Belgium (5), the United States (11), Malaysia (3), South Africa (25), Italy (31), Holland (38), and Germany (38); in guinea fowl with transmissible enteritis in Italy (23, 31) and France (2); and in partridges (13) and pheasants (12) in the United Kingdom. In addition, ELVs have been found in feces and enterocytes of cockatoos and galahs with enteric disease in Australia (30, 44) and in the gut contents of ostriches showing signs of enteritis in South Africa (8). Based on these findings, it is likely that avian ELVs have a worldwide distribution.

Natural and Experimental Hosts

Infections with ELVs have been described in turkeys, chickens, guinea fowl, partridges, pheasants, ostriches, and psittacine species. The majority of naturally occurring infections in domestic poultry have been identified in young birds during the first few weeks of life. However, a chicken ELV was isolated from the meconium of a dead-in-shell chicken embryo (39), indicating that infection with these viruses may occur in adulthood.

Transmission, Carriers, and Vectors

The principal site of replication of ELVs is the small intestinal epithelium (Fig. 12.16); some chicken ELVs also replicate in the kidney (37). Thus, infection most likely is spread horizontally through ingestion of infected feces, but other routes of spread cannot be ruled out. Isolation of a chicken ELV from meconium of a dead-in-shell chicken embryo indicates that this virus is vertically transmitted (39); it is likely that other ELVs also are transmitted in this manner. Additionally, evidence indicates darkling beetle larvae may act as mechanical vectors for turkey ELVs (7).

Clinical Signs

The main clinical signs associated with naturally occurring ELV infections in domestic poultry are diarrhea, decreased feed efficiency, and uneven growth. Increased mortality also may occur. Disease is most frequently seen in birds in the first few weeks of life. Enteric disease has been reproduced experimentally in neonatal birds inoculated orally with ELVs.

In 3- or 4-day-old specific-pathogen-free turkeys experimentally infected with a U.S. turkey ELV, depression, watery droppings, and pasted vents were observed. Signs were first evident 3–4 days PI. Similar signs occurred in poults inoculated at 2, 3, and 4 weeks of age. Decreases in body weight gain also were observed. ELVs were detected in greatest numbers in the intestinal

contents of inoculated turkeys at 3 and 4 days PI but were present in some birds up to 14 days PI (17, 19, 41).

Abnormal feces and transient stunting of growth of variable severity were observed in broiler chickens dosed orally with ELVs (5, 25, 29). Specific-pathogen-free chicks inoculated orally with Japanese ELVs showed diarrhea and variable mortality (up to 53.3%), dying between 2 and 6 days PI (37).

An ELV isolated from guinea fowl with transmissible enteritis in Italy suppressed weight gains of commercial guinea fowl when they were inoculated orally at 1 day of age (31).

A naturally occurring syndrome associated with ELV infection in young galahs and sulfur-crested cockatoos was characterized by intractable profuse diarrhea, wasting, and death (30, 44).

Pathology

Gross lesions in turkeys experimentally infected with a U.S. turkey ELV consisted of thin-walled, dilated ceca filled with yellow, foamy fluid and extreme paleness of the serosa of the gastrointestinal tract; catarrhal secretions were detected in the small intestines. Morphometric studies indicated varying degrees of shortening of the villi and elongation of crypts along the length of the small intestine (17, 19, 41). In naturally occurring infections in turkeys, ELVs usually occur as a component of mixed infections. Interestingly, poults experimentally infected with a combined turkey ELV/group A rotavirus inoculum were more severely affected in terms of clinical signs, body weight gain, and lesion severity than poults that received either inoculum alone (17).

Chicks experimentally infected with Japanese ELVs and that died 2–6 days after inoculation showed microscopic changes characteristic of baby chick nephropathy (i.e. nephrosis and visceral urate deposition) (37). Chicks experimentally infected with ELVs from broilers in Belgium with a runting syndrome had pale small intestines with watery and sometimes filamentous contents of the small intestine and ceca (5).

In a naturally occurring syndrome associated with ELV infection in young galahs and sulfur crested cockatoos, the intestine was dilated with mucoid fluid and gas, and the walls appeared thickened. Microscopic lesions in the intestine consisted of villus atrophy and fusion, elongation of the crypts of Leiberkuhn, marked epithelial cell proliferation in the crypts and shortened villi, with inflammation of varying severity (30, 44).

Thin section EM has revealed the presence of intracytoplasmic, crystalline arrays of particles resembling enteroviruses in the enterocytes of infected chickens (6, 27) and cockatoos (30, 44).

Pathogenesis of the Infectious Process

The nature of the microscopic lesions in the small intestine of affected birds suggests that infections with ELVs produce malabsorption and diarrhea due to destruction of small intestinal villus epithelial cells. Measurement of absorption of D-xylose from the intestines of experimentally infected turkey poults confirmed that a transient malabsorption was present in poults inoculated at 3 days of age, but not in poults inoculated at 2 weeks of age (17). However, it also has been suggested that the turkey ELV exerts its effects by altering the cellular physiology of the villus epithelial cells, altering the normal intestinal flora, or through a systemic mechanism (as evidenced by a transient lymphopenia) (41).

Replication in other organs, such as the kidneys, also may contribute to the pathogenesis of these viruses.

Immunity

The development of active immunity to ELV infections has not been investigated. Similarly, the extent to which passively acquired maternal antibodies provide protection from these infections is unknown.

Diagnosis

Isolation and Identification of Causative Agent

Diagnosis of ELV infections in avian species most commonly is accomplished by EM examination of droppings or intestinal samples. ELVs have been identified using both direct and immune EM procedures. For direct EM, droppings or intestinal contents are prepared as suspensions (10–20%) in phosphate-buffered saline and centrifuged at 800 x g for 20 minutes to remove large particulate material. The supernatant fluid subsequently is centrifuged at 15,000 x g for 20 minutes in a benchtop centrifuge, and the resultant pellet is resuspended in approximately 500 μL distilled water and 100 μL 2% phosphotungstic acid. After mixing, the material either is sprayed onto formvar-filmed copper grids, or a drop of the material is placed on the grid for 1–3 minutes and removed by blotting on bibulous paper. ELVs also may be detected in droppings or intestinal contents using immune EM (34); however, this procedure requires availability of specific antisera.

Confirmation that particles observed by EM are animal viruses is achieved by isolating the viruses in turkey or chicken embryos or in cell cultures as described previously. Antigenic characterization of the isolate is dependent on the availability of serogroup-specific antisera. Yolk sac membranes or chorioallantoic membranes from inoculated embryos may be prepared as impression smears or cryostat sections and examined by immunofluorescent staining using serogroup-specific antisera. This serologic procedure will distinguish between isolates of known serogroups and aid in the identification of new serogroups.

An antigen-capture enzyme-linked immunosorbent assay (ELISA) was described for detection of turkey ELV in turkey intestinal contents (16). The procedure was shown to be a rapid, highly sensitive, and specific method for diagnosis of the virus.

Serology

Antibodies to ELVs have been detected by serum-neutralization and indirect immunofluorescence tests (6, 29, 37); however, because virus isolates and reference antisera are not widely distributed, routine serologic diagnosis is not recommended. However, serology is useful to determine the status of specific-pathogen-free birds with respect to ELV infections.

Differential Diagnosis

Enteric disease associated with ELVs needs to be distinguished from similar conditions caused by other enteric viruses, such as

rotavirus, astrovirus, and coronavirus; clinical signs and lesions are not pathognomonic. However, mixed infections of ELVs and other enteropathogens occur commonly, and it may be difficult to identify the relative importance of each constituent of mixed infections.

Intervention Strategies

The role of ELVs as avian pathogens has not yet been fully defined; consequently, no specific therapeutic or prophylactic measures are available. Given the importance of some of the conditions with which avian ELVs have been associated, it would be prudent to develop better diagnostic methods for these viruses in order to investigate their epizootiology and pathogenicity more fully.

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Turkey Torovirus Infection

D. L. Reynolds and A. Ali

Introduction

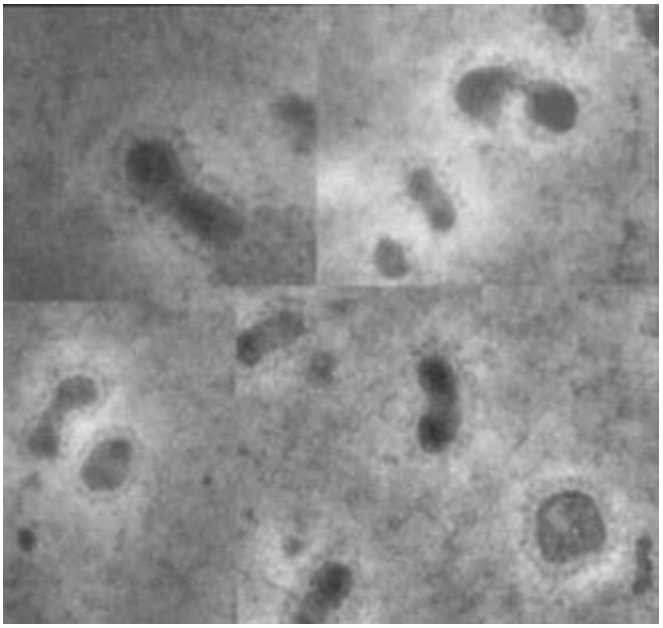
Toroviruses are classified in the order Nidovirales and the genus Torovirus of the family Coronaviridae (13, 15). Comparative genomic analysis has provided evidence to suggest that toroviruses should be a subfamily of Coronaviridae or perhaps their own family within the Nidovirus order (14). The first torovirus to be classified, the Berne virus, was isolated and identified from a horse during the early 1980s and is the only torovirus that has been propagated in cell culture (equine dermal cells) (15, 33). Since then, toroviruses have been detected in cases of diarrhea / enteritis from humans, dogs, cattle (Bovine breda 1 and breda 2 viruses) and pigs (12, 16–19, 23, 24, 28, 29, 34). More recently, an enteric virus formerly known as stunting syndrome agent (SSA) has been detected in young turkeys with diarrhea (2). This virus now has been determined to be a torovirus. The relationship between the turkey toroviruses and toroviruses from other animal species have not been determined. Additionally, the economic impact of torovirus infection(s) on the turkey industry has not been established.

History

Stunting syndrome (SS) is a condition of young poultry characterized by diarrhea, poor weight gain, and suboptimal performance (11, 22, 24, 35). In poults, the condition has also been referred to as malabsorption syndrome, poult enteritis, turkey viral enteritis, etc. (24). A number of infectious agents (including viruses, bacteria, and parasites) and noninfectious etiologies have been associated with this condition (24). In the early 1990s, the disease was reproduced in turkeys under laboratory conditions. Subsequently, a disease model was developed using an inoculum containing the etiologic agent (now known as turkey torovirus) (10). Using this laboratory model, stunting syndrome of turkeys was characterized. It was found that poults with stunting syndrome had decreased weight gain, diarrhea, decreased disaccharidase activity, and poor feed conversion (9–11). The etiologic agent was isolated and identified several years following the characterization of the disease (2). Stunting syndrome of turkeys is caused by a virus initially referred to as the stunting syndrome agent (SSA). Further characterization of the SSA revealed physicochemical properties consistent with toroviruses (5). Additionally, it was found that the SSA was antigenically and genomically different from other known avian pathogens including enteric coronavirus, infectious bronchitis, and Newcastle disease virus (6). It was revealed that the SSA genomic sequence is very similar to the polymerase protein gene of Berne virus—a member of the genus Torovirus (Ali and Reynolds, unpublished data). Based on the physicochemical properties and this recent genomic sequence information, the SSA is being referred to as turkey torovirus.

Incidence and Distribution

Little information is available concerning the prevalence of torovirus in turkeys and other avian species. A direct and indirect immunofluorescence assay to detect toroviral antigens (in the intestinal tissue) and antitorovirus serum antibodies, respectively, was developed (27) and was used in a survey of turkey flocks in



12.17. Composite of four electronmicrographs of turkey torovirus. Note the virus envelope with peplomeres and the various morphology of the nucleocapsid that is dependent on its orientation. Size of particles vary from 60 to 95 nm.



12.18. The intestinal tracts of 27-day-old turkey embryos (just prior to hatch). The torovirus infected intestine is dilated and filled with fluid containing torovirus. (*Avian Diseases*)

the United States (26). The results of this study indicated that about 30% of turkeys experiencing enteric disease (i.e., stunting syndrome) were positive for torovirus. Additionally, antitorovirus serum antibodies were detected from turkey flocks in Israel. These results provided evidence that turkey torovirus occurs in other countries and continents beyond the United States.

The enteric disease caused by turkey torovirus infection usually occurs during the first 3 weeks of life in turkey poults. The older turkeys are susceptible to infection, but the weight loss and enteric disease is mild or asymptomatic.

At present, it is not known whether torovirus infected turkeys become carriers and shed virus following infection or whether egg transmission occurs. Additionally, the mechanism(s) by which the turkey torovirus is maintained in the environment is also not known. Chickens and chicken embryos are resistant to infection with turkey torovirus.

Etiology

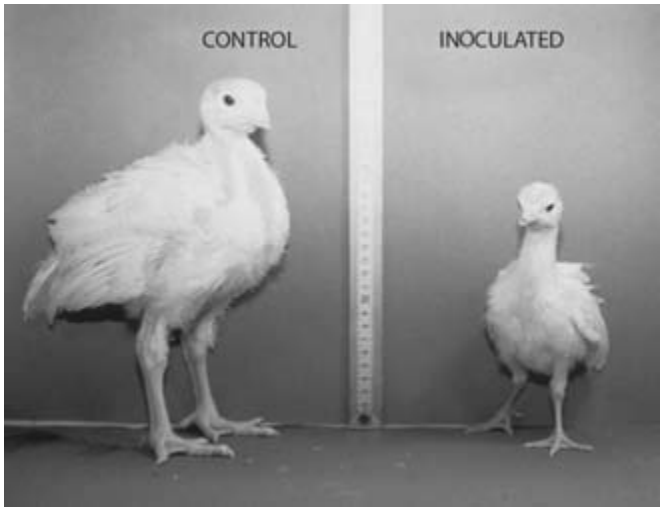
Turkey torovirus is an enveloped virus that is about 60–95 nm in diameter when examined with transmission electron microscope (2). Morphologically, the nucleocapsid appears as dumbbell-shaped, kidney-shaped, or comma-shaped depending on its orientation and is surrounded with a membrane containing peplomers (Fig. 12.17).

Turkey torovirus agglutinated rat erythrocytes at 4°C and at room temperature but did not agglutinate erythrocytes from chickens, turkeys, rabbits, mice, guinea pig, cats, horses, dogs, sheep, and cattle. Turkey torovirus lost its infectivity after treatment with ether but was stable at acidic pH (pH 3.0) for 1 hour at 37°C. The virus is inactivated at pH 10.0. Turkey torovirus was resistant to treatment with sodium deoxycholate and phospholipase C. Treatment of turkey torovirus with trypsin, chymotrypsin, and pancreatin resulted in the enhancement of viral infectivity. The purified viral genome is sensitive to treatment with RNase enzymes and appears to be polyadenylated at its 3′ end (5). Partial sequence data of the turkey torovirus genome indicated that it is related with the polymerase protein gene of Berne virus, a member of the genus Torovirus (Ali and Reynolds, unpublished data).

Turkey torovirus was propagated in primary culture of turkey intestinal epithelial cells (1, 2). The virus can also be propagated in 23–24-day-old turkey embryos when inoculated via the amniotic route (3). Torovirus replication in turkey embryos was limited to the intestinal tract. Virus replication in the intestines was detected by immunofluorescence and reverse transcription-polymerase chain reaction (RT-PCR). Torovirus replication in embryos causes accumulation of excessive amounts of fluid within the lumen of the intestine (Fig. 12.18), decrease in intestinal maltase activity and a decrease in the absorption of D-xylose by the intestine.

Pathogenesis and Epidemiology

Turkey torovirus is known to infect turkeys. Chickens are refractory to infection with the virus. The clinical disease caused by torovirus is observed during the first few weeks of life. Turkeys older than 4 weeks are infected with torovirus, but clinical disease is mild or asymptomatic. In torovirus-infected poults, the duration of clinical signs is usually 7–10 days and include diarrhea (loose, frothy droppings containing undigested feed material), listlessness, and litter eating. Decreased weight gain(stunting) and poor feed conversion are of major concern and of economic significance (11). Mortality is generally low, but morbidity is high. Torovirus infected turkey flocks appear uneven in size (Fig. 12.19)



12.19. Turkey poults from the same hatch that had been reared identically. The smaller poult displays the effect of stunting syndrome (i.e. torovirus infection). (Courtesy of Jerry Sell, Iowa State University)

and do not achieve weight gains comparable to uninfected flocks. The intestinal disaccharidase activity and intestinal D-xylose absorption is reduced in infected poults. Experimental studies with turkey torovirus have shown that oral inoculation of day old poults with a gradient purified preparation of virus results in decreased weight gain, diarrhea, decreased intestinal disaccharidase(e.g., maltase, sucrase, etc.) activity and decreased D-xylose absorption (2). The decrease in intestinal maltase activity is detectable at 2–3 days post inoculation and persists for 10–14 days post inoculation. The intestines appear thin-walled and pale. Intestinal contents are watery and contain undigested feed material. The ceca are dilated, and cecal contents are watery, frothy, and brownish in appearance. The disease in poults inoculated with pure virus is of shorter duration and less severe than in poults inoculated with homogenates prepared from the intestines of infected poults. This provided evidence that other factors (i.e., bacteria and other microbes) contribute to the disease (31). Inoculation of turkey embryos (at 23–24 days of embryonation) via the amniotic route with torovirus causes disease that has similarities to natural torovirus infections in poults. Excessive fluid accumulation in the intestines (Fig. 12.18), decreased intestinal maltase activity, and decreased D-xylose absorption are typical (3). Torovirus infected embryonic intestines were thin-walled, pale, and fragile. The intestines were filled with pale-yellow to greenish fluid containing torovirus. The amount of fluid within the intestine increased with time following virus inoculation. Histologically, the changes in the intestinal cells were subtle and may not always be evident. As stated previously, diarrhea was observed in young poults infected with turkey torovirus. Similarly, turkey embryos infected with torovirus exhibited excessive fluid accumulation in the intestines. This fluid accumulation is measured easily (by weighing) and is a useful indicator for quantitating diarrhea. The mechanism(s) of this fluid loss into the intestine by poults and embryos is not understood. One plausible mechanism of diarrhea from torovirus infection may be from the damage induced to the intestinal epithelium that leads to the accumulation of undigested and unabsorbed feed in the intestinal lumen. This, in turn, can cause an osmotic effect on the movement of water across the intestines leading to fluid loss. It should be noted, however, that intestines observed from embryos inoculated with turkey torovirus exhibit minimal (if any) damage to the intestinal epithelium (i.e., histologically, the intestines appear normal). Additionally, feed material is absent in the intestinal lumen in embryos so there can be no osmotic effect. These observations suggest that an additional mechanism(s) may be involved in fluid loss during turkey torovirus infection. Previous work suggests that immune cells or their products may contribute to the fluid loss during infection with turkey torovirus (4). When turkey embryos were treated with cyclophosphamide to deplete them of their immune cells and these immune cell depleted embryos were infected with turkey torovirus, the embryos secreted significantly less fluid within their intestines than torovirus infected embryos that had intact (normal) immune cells. Additionally, it was also observed that the intestinal epithelial cells from infected embryos had higher levels of mRNA for pro-inflammatory cytokines including interleukin-8, tumor necrosis factor, and macrophages-monocytes inflammatory protein than uninfected normal controls (7). These observations suggest that immune cells are likely to be attracted to the site of virus insult (i.e., the intestine) and these cells or their products play a role in the pathophysiology of torovirus enteric disease. Furthermore, infection of turkey poults and embryos with turkey torovirus lead to activation (increased metabolic activities and increased level of serine esterases-enzymes found in higher concentrations in the cytoplasm of activated cytotoxic lymphocytes) of intestinal intraepithelial lymphocytes. These results further suggest that immune cells, including the intestinal intraepithelial lymphocytes, are activated during infection with turkey torovirus and may contribute to the diarrhea/fluid loss during infection.

The intestinal disaccharidases (e.g., sucrase, maltase, etc.) are also reduced during infection with turkey torovirus in turkey embryos as well as poults. The active disaccharidase enzymes are normally present in the brush border membrane of mature intestinal epithelial cells found at the tip of the villous (30). These enzymes are required for end-stage digestion of disaccharides into final products (respective monosaccharides) before absorption occurs by the epithelial cells. The mechanism(s) of this decrease in enzymatic activity is not known. There is little, or no, loss of mature intestinal epithelial cells during infection with turkey torovirus. During experimental infection of turkey embryos with torovirus, the epithelial cells from control and infected embryos had similar levels of mRNA for sucrase-isomaltase enzyme complex when examined by competitive RT-PCR (4). This observation suggests that the mechanism(s) for the decrease in disaccharidase activity is beyond the post transcriptional level. The mechanism(s) of this blockage is not known. Some lymphokines have been reported to influence the activity of disaccharidases in some cells (20, 21). During our studies where immune cell-deficient turkey embryos (following treatment with cyclophosphamide) were inoculated with the turkey torovirus, there was not a signif-

icant decline in intestinal maltase activity following infection despite virus replication in the intestinal epithelium. It was assumed from these observations that immune cell(s) or their products may also play a role in the mechanism(s) responsible for decreased intestinal maltase activity that was observed following viral infection.

The presence of bacteria in the inoculum containing enteric viral pathogens including turkey torovirus has been reported to increase the severity and duration of enteric disease following infection (31). The identity of bacteria that may be involved in increasing the severity of enteric disease, and the mechanism(s) responsible for their additive effect, are unclear (8). It is also not known whether there is a synergistic effect(s) between other enteric agents (i.e., viruses, parasites, etc.) on the outcome of enteric disease.

Diagnosis

Diagnosis of turkey torovirus infection can be achieved by a number of methods. Direct electron microscopic examination of the cecal contents may be used. However, a number of factors need to be considered when using this technique. Torovirus is a membraned virus; therefore, freezing and thawing of clinical specimens (i.e., intestinal tracts and or feces) generally result in a loss of peplomers, and identification and differentiation (from cell membrane debris) become difficult. Torovirus associates with intestinal epithelial cells and its demonstration from intestines/feces may require concentration from large volumes of clinical specimens. The virus was demonstrated(from 3–9 days post inoculation) in lysed intestinal epithelial cells isolated from infected poults (2). Toroviral antigens were demonstrated from the intestinal epithelium by direct fluorescent antibody (FA) assay (27). The cells containing viral antigens were observed along the middle portion of the villous. The toroviral antigens were demonstrated in the intestinal epithelial cells from 2–12 days postinoculation. Torovirus infections can also be diagnosed by detecting serum antibodies using the indirect fluorescent antibody (IFA) assay. While developing the IFA assay, infected poults were a better source of viral antigen for IFA than infected embryos because the nonspecific fluorescence (i.e., background) was less when using intestines from infected poults than from infected embryos. Turkey torovirus specific antibodies were demonstrated as early as 5 days postinoculation by IFA under experimental conditions. Torovirus specific antibodies have been detected in sera from 25-week-old turkey flocks in field cases.

A reverse transcription-polymerase chain reaction (RT-PCR) assay was developed for turkey torovirus and was used to detect torovirus under experimental conditions (Ali and Reynolds, unpublished data). Further development of this assay may allow for its use in field conditions. Turkey torovirus can be isolated by inoculating turkey embryos (23–24-day old) via the amniotic route (3). The inoculated embryos are incubated for 96 hours and observed for lesions (fluid accumulation in the intestines) and decreased intestinal maltase activity. The torovirus infected intestines (and contents) may need to be blindly passaged a few times in turkey embryos to observe the lesions. The intestinal fluid contains torovirus that can be visualized by direct EM examination. Although cultured, primary intestinal epithelial cells are susceptible to virus replication, these cells may be of limited use for routine isolation of turkey torovirus because of difficulties in maintaining these cells *in vitro* and their extraordinary susceptibility to toxic substances within the inoculum (1).

Treatment, Prevention, and Control

There are no vaccines available to control or prevent infection or disease induced by turkey torovirus. Additionally, because the disease usually occurs within the first 1–2 weeks of life, vaccination after hatching may not have ample time to generate a protective immune response. Age susceptibility with torovirus infection in turkey poults exists (see previous discussion). That is, birds infected within the first few days following hatch develop the most severe form of the disease; whereas, birds infected later in life (i.e., after 4 weeks of age) develop less severe clinical disease or may be asymptomatic. Therefore, prevention and control strategies that are targeted for early protection and prevention will have the greatest impact. Under experimental conditions, the practice of providing artificial passive immunity has proved to be beneficial (25). Poults that received anti-turkey-torovirus antibodies at the day of hatch and were challenged a day later with torovirus gained significantly more weight than challenged poults inoculated with nonspecific antibodies or challenged poults that received no antibodies. The passively administered antibodies were unable to prevent torovirus infection, but the severity and duration of the disease were greatly reduced. Such studies suggest that passive immunity either via the breeder hen vaccination or by artificial means may be a feasible approach for the prevention or treatment of torovirus disease. Good management practices to reduce or eliminate the exposure of poults to torovirus should be followed. The use of enteric antibiotics to lessen the severity and duration of stunting syndrome has been reported (32). Additionally, the use of specific diets or dietary components has been reported to provide beneficial effects (9). It should be noted that these studies were performed with inoculum that contained torovirus and other undefined agents (i.e., bacteria, etc.). The effects of these treatment regiments may not have been on torovirus but most likely on bacteria or other factors that may have contributed to the clinical disease.

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Chapter 13

Viral Infections of Waterfowl

Introduction

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The objective of this chapter is to bring together under one heading the viral diseases that affect waterfowl. This chapter has details on the following diseases:

- Duck hepatitis, a major disease of young ducklings, caused by three different viruses, duck hepatitis virus (DHV) types 1, 2, and 3. Since the last edition new information on the molecular biology of DHV-1 has been reported.
- Duck virus enteritis (DVE), a potential threat to both commercially reared and wild waterfowl. Advances made in the detection of the virus by the use of the polymerase chain reaction (PCR) technique (25), (52) (54), (77) should be a great aid to rapid diagnosis of the disease, particularly in wild and ornamental collections. The use of PCR may also result in a greater understanding of the pathogenesis and possibly latency of this herpesvirus.
- Parvovirus infections of geese and Muscovy ducks. Molecular techniques have shown that although MDPV and goose parvovirus (GPV) are closely related, they are also distinct and, therefore, should be considered as causing separate diseases. Muscovy duck parvovirus (MDPV) has been recognized in the United States (76).
- Goose hemorrhagic polyomavirus (GHPV) is the cause of hemorrhagic nephritis enteritis of geese (HNEG) (24).
- Other virus infections associated with waterfowl will be covered in this Introduction. Viruses that are not necessarily associated with disease in waterfowl but still infect these species will also be included.

Interests in viral infections of waterfowl are more diverse than for poultry and focus on 3 different issues: a) problems associated with raising birds commercially; b) problems associated with wild waterfowl; and c) problems created by migratory waterfowl in the transmission of infectious disease to commercially reared waterfowl and poultry.

RNA viruses associated with waterfowl include Picornaviridae (DHV I & III), Astroviridae (DHV II), Paramyxoviridae (avian metapneumovirus, avian paramyxoviruses), Orthomyxoviridae (avian influenza), Flaviviridae (West Nile virus), and Reoviridae (Muscovy duck reovirus and goose reovirus); whereas DNA viruses include Herpesviridae (DVE, GHV), Adenoviridae (duck adenovirus), Circoviridae (circovirus-like infection of ducks and geese), Hepadnaviridae (duck hepatitis B virus), Parvoviridae (MDPV and GPV) and Polyomaviridae (GHPV).

Oncogenic viruses of waterfowl are not included in this chapter.

Avian Paramyxoviruses

Avian paramyxoviruses (APMV) types 1, 4, 6, 8 and 9 have been associated with infections in waterfowl, but generally are considered to be apathogenic. Virus isolations from waterfowl have been reported (23), (55), (60) (58), (26), (30), (13). Antibody responses in waterfowl to various AMPVs have also been reported (9), (10), (29), (47).

Chang *et al.* 2001 (14) reported on the complete nucleotide sequencing of a APMV 6 isolated from ducks, that was apathogenic.

An exception to this are reports by Zou *et al*. 2005 (78) and Jinding *et al*. 2005 (36) of an APMV type 1 causing high mortality in geese in China.

APMVs are dealt with in detail in Chapter 3.

Avian Metapneumovirus

Avian metapneumoviruses (aMPV), a relatively newly described group of viruses in the family Paramyxoviridae (53) were first isolated in 1980's (21). They have been of most concern to the turkey industry and were not detected in the United States until 1996 (59). The US turkey isolate of aMPV is designated type C, as it is different from the types A and B found in Europe (57).The isolates from ducks in France (73) have also been assigned to subgroup C and Toquin *et al.* (2006) (74) has shown that the European and American subgroup C viruses belong to different genetic lineages.

In 1999, Toquin *et al.* (73) reported the isolation of a pneumovirus from 42-week-old Muscovy ducks exhibiting coughing for 7 days followed 2 weeks later by an egg drop. Mortality was about 2%. Grossly, the birds showed general congestion and splenomegaly; histologically, tracheitis was also observed. The

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virus was isolated from tracheal swabs using Vero cells. After one passage, a cytopathic effect characterized by distended cells detaching from the monolayer and syncytia was observed. Indirect immunofluorescence staining of the culture was positive with a polyvalent antipneumovirus serum prepared in SPF turkeys that had been inoculated simultaneously with 4 aMPV strains. The isolate was confirmed as a pneumovirus by a reverse-transcriptase polymerase chain reaction (RT-PCR) focused on the N gene, which is diagnostic for aMPV, irrespective of the virus subgroup. The authors also showed that using monospecific aMPV antisera in both an ELISA and an indirect IFA, the Muscovy duck virus isolated reacted most strongly with the aMPV Colorado antiserum when compared with antisera against aMPV A, B, and non A-non B. The closeness to the Colorado virus strain was confirmed by RT-PCR because the isolate did not react with G-based primers specific to subgroups A & B. The pathogenicity of the aMPV isolated required further investigation to determine its role in this syndrome because *Reimerella anatipestifer* was also recovered. Jestin *et al*. (35) subsequently has reported that experimental infection of 18-day-old SPF Muscovy ducklings with the aMPV isolate did produce clinical signs that could be correlated with virus isolation from tracheal swabs and preceded any seroconversion.

aMPV RNA has been isolated from the nasal turbinates or swabs of mallard ducks, wild geese and sentinels captured in the north central US (63). The nucleotide and amino acid sequences of the aMPV genes examined had 90–95% identity with isolates from turkeys. It is not clear whether virus was isolated in all these cases. The authors considered that wild birds may well be involved in the spread of the disease in turkeys. McComb *et al.* (45) reported the detection of aMPV viral RNA in choanal-tracheal swabs collected from 8-week-old sentinel ducks (mallards) that were allowed to mingle with wild waterfowl in central Minnesota. In one case, aMPV was isolated from the choanal-tracheal swabs, and the virus had more than 96% predicted amino acid identity with the MN/2A turkey aMPV isolate. The authors (45) also detected aMPV viral RNA in choanal swabs from Canada geese, blue winged teal, and snow geese; however, no viruses were isolated from these birds. They concluded that aMPVs may be more widespread in nature than previously thought.

In an experimental study (48) 10-day-old commercial ducklings were inoculated either orally or oculonasally with a Minnesota turkey isolate of aMPV. The birds remained clinically normal, but at 3–21 days PI, aMPV viral RNA was detected in tissues from inoculated birds. aMPV was also reisolated from the infected ducklings.

aMPV-negative mallard ducks placed next to a turkey farm experiencing a severe outbreak of aMPV infection did not develop clinical disease but infectious aMPV was recovered from choanal swabs after 2 weeks and anti-aMPV antibodies were detected after 4 weeks. This indicated that the aMPV isolates from turkeys and ducks shared a common source and viruses from different species can cross-infect. (61)

Bennett *et al.* 2002 (7) demonstrated the presence of aMPV in wild Canada geese (*Branta canadensis*) and blue winged teal (*Anas discors*) by RT-PCR. By virus isolation aMPV could only be recovered in Canada geese. Thus RT-PCR appears to be more sensitive in detecting aMPV. Using Canada geese isolates Bennett *et al*. 2005 (6) went on to investigate the genomic structure of the virus. All but one of the eight genes were similar to those of a turkey aMPV isolate in terms of size, sequence identity and genome organization. However the attachment gene (G) coding for a 585 aa protein was larger than for any pneumovirus or metapneumovirus, being more than twice the size of G proteins for other subtype C viruses and human metapneumoviruses and more than 170 amino acids larger than the G proteins from aMPV subtypes A, B and D. Although this virus with the large insertion in the G gene was not detected in turkeys, it did replicate in the upper respiratory tract of experimentally challenged domestic turkeys with no clinical signs. This virus could also be horizontally transmitted to naïve turkeys and the infection could induce specific antibody production. The authors suggested that this virus may be a safe and effective vaccine for commercial turkeys.

Shin *et al.* 2001(62) considered ducks could be suspected to play a role as nonclinical carriers of aMPV and serve as a potential source of infection for domestic turkeys.

Avian Influenza Virus

Until recently information on avian influenza in waterfowl was reported as follows: Waterfowl typically do not experience significant disease problems due to avian influenza viruses (AIVs), but infections in these birds are widespread (71). AIVs can be recovered from migratory waterfowl, particularly ducks; at least 30 of 149 species of ducks, geese, and swans have yielded virus but natural infections are usually considered asymptomatic(72).

The picture changed dramatically in late 2002 when HPAI (H5N1) occurred in geese, ducks and swans among other avian species at two waterfowl parks in Hong Kong (18). The range of pathological changes present in the various waterfowl examined in this outbreak resembled those reported generally for HPAI viruses in chickens (72).

Since that outbreak in 2002 H5N1 HPAI, also referred to as "bird flu" has spread through Asia, Europe and parts of Africa.

The role of migratory waterfowl in the spread of H5N1 HPAI has been questioned. Chen *et al.* 2005 (16) described an outbreak in bar headed geese (*Anser indicus*) at Qinghai Lake in Western China in May 2005; more than 1500 birds died.

Hulse-Post *et al.* 2005 (34) reported that the H5N1 HPAI can revert to non-pathogenicity in ducks. Thus wild waterfowl including ducks may appear uninfected by the H5N1 but still shed virus and so continue to circulate the virus. Similar findings were reported by Sturm-Ramirez *et al.* 2005 (69).

There are many reports about the H5N1 HPAI and the role of waterfowl in its transmission; this virus is covered in detail in Chapter 5.

West Nile Virus

West Nile virus (WNV) is a member of the Japanese encephalitis virus antigenic complex of arthropod-borne flaviviruses

(Flaviviridae) that are transmitted through mosquitoes to a variety of mammals and birds (39). The notoriety of this virus has increased since 1999 when an epizootic causing deaths in wild American crows (*Corvus brachyrhynchus)* began in New York (50). This was the first time that WNV had been detected in North America. At the same time, WNV-positive cases occurred in a number of wild bird species, humans, and horses (39, 40, 68) as well as a zoological collection of mammals and birds in New York (12).

Outbreaks of WNV involving ducks and geese have been reported from Israel and Romania (40, 49, 56). In the New York outbreak, Steele *et al.* (68) examined birds from two wildlife facilities in the city, which had either died or were euthanatized and were suspected of being infected with WNV. Lesions seen in emaciated Anseriformes included brain hemorrhages, splenitis, splenomegaly, nephritis, and congestion in the kidneys. Three ducks were involved in this study, 1 mallard and 2 bronze-winged ducks. In the mallard, abundant antigen was demonstrated by immunohistochemistry in the brain, heart, liver, kidney, and pancreas and to a lesser extent in the adrenal gland and intestine. Virus in excess of 10^2 pfu/0.2 ml was isolated from brain, heart, spleen, liver, and kidney; virus was also demonstrated by RT-PCR in these same tissues. Only immunohistochemical testing was performed on the tissues from the bronze-winged ducks, but results obtained were not dissimilar to those in the mallard.

Calle *et al.* (12) reported that in the 1999 outbreak in New York an Abyssinian blue-winged goose (*Cyanochen cyanopterus*), a roseybill duck (*Netta peposcaca*), and a domestic goose (*Anser anser*) showed asymptomatic seroconversion to WNV, and in the domestic goose and trumpeter swan (*Cygnus cygnus buccinator*) morbidity and recovery were recorded. No deaths were recorded in any of the birds.

The New York isolates of WNV yielded an E gene nucleotide sequence that was extremely homologous to that from the WNV isolated from a goose in Israel in 1998 and also from a 1996 Romanian isolate(40, 42).

In an experimental study, Swayne *et al*. (70) reported WNV replication in 2-week-old goslings following s/c inoculation. A viremia was detected between 1–5 days PI; virus could be detected in plasma diluted up to $1{:}10^6$. All the experimentally infected goslings developed clinical signs, typified by depression, weight loss, and decreased activity; one bird developed neurological signs. Microscopic lesions seen in the infected birds were encephalitis and myocarditis. Virus was recovered from the brain and the heart. Viral antigen was demonstrated by immunohistochemistry in the heart, brain, pancreas, and kidney and in autonomic ganglion cells of the intestine (varied from bird to bird). These authors concluded that the disease was a threat to goslings; the high virus titers in the blood suggested that goslings could be an amplifying host and, thus, infect permissive mosquito vectors; the virus was transmitted by contact between goslings in close association.

Bird to bird transmission of West Nile virus in geese has been proposed by Austin *et al.* 2004.(1) They investigated an outbreak on a multiage farm with multiage flocks in Manitoba, Canada. The level of seroconversion in these flocks exceeded that which would have been expected by mosquito transmission alone. In another study Banet-Noach *et al*. (4) showed direct (non-vector) transmission of WNV in geese. They mixed a group of 10, 3-week-old s/c inoculated geese with 20 healthy geese of the same age in an insect-proof room. All geese in the inoculated group produced antibodies, 8 became viremic and 5 died between 7–10 days PI. Virus was shed via the cloaca and oral cavity by 3 geese. Two of the contact birds died on days 10 and 17 pi and WNV was recovered from another 3 birds. The authors claim these findings strongly suggest that horizontal transmission can occur in commercial flocks and may be aggravated if cannibalism and feather picking of sick birds occurs.

RT-PCR procedures for detecting WNV have been described (8, 40, 56).

Muscovy Duck and Goose Reoviruses

Muscovy duck reovirus infections have been described (37, 43). In France, it is considered to be a major virus disease of Muscovy ducks (41). In 2-4-week-old ducks, the disease is acute; morbidity is high; and mortality can reach 10%. Clinical signs include apathy, with diarrhea and difficulties in moving. Grossly, dead birds showed fibrinous pericarditis, marbled spleen, and enlarged friable livers (43, 44). Microscopically, the pericarditis consisted of an infiltration of mononuclear inflammatory cells in the serous membrane associated with a fibrinous exudate. In the liver, there were necrotic foci or infiltrations of lymphocytes and plasmacytes in the portal region. In the spleen, reticulosis, depletion of lymphoid nodules, and focal necrosis were evident. The synovial sheath of the leg tendons had an exudative inflammation (44).

Cross neutralization tests have demonstrated the Muscovy duck reovirus to be antigenically distinct from the chicken reovirus S1133 (28).

A reovirus has also been reported recovered from a mallard (46).

Palya *et al*. 2003 (51) reported on a disease in young geese caused by a reovirus. The disease was characterized by splenitis and hepatitis with miliary necrotic foci during the acute phase and epicarditis, arthritis and tenosynovitis during the subacute/chronic phase. Although in earlier reports reoviruses had been repeatedly recovered from geese they had never been associated with disease. The disease occurred most frequently in 2–20-week-old goslings and Banyai *et al.* 2005 (5) investigated the genetic variability among goose reoviruses (GRV). The S4 genome segment of five GRVs shared substantial structural similarity with Muscovy duck reovirus (DRV). The authors consider GRV and DRV to belong to a species distinct from others established within the subgroup 2 of orthoreoviruses.

Hollmen *et al.* 2002 (31) reported the isolation of a reovirus from common eider ducks (*Somateria mollissima)* in Finland. The virus was isolated from the bursa of Fabricius in Muscovy duck embryo fibroblasts, but not in SPF chicken eggs. Mallard ducklings challenged with this virus seroconverted. No deaths were recorded but at necropsy focal hemorrhages were observed in the liver, spleen and bursa of Fabricius of some of the birds. The polyvalent eider reovirus serum obtained from the experi-

mental mallards did not inhibit the growth of avian arthritis virus (S1133) in Muscovy duck embryo fibroblasts. The relationship of this reovirus to DRV and GRV has not been reported.

Goose Herpesvirus (GHV)

In Australia, a herpesvirus has been implicated in a peracute disease of domestic geese that caused 97% mortality over a 24-day period (38). Clinical signs and gross pathology were similar to those seen with DVE infections. Histologically small button ulcers and large plaques overlying lymphocyte aggregates were present on the mucosa of the small intestine of affected birds. Small white foci of necrosis and focal hemorrhages were seen in the livers. Numerous intranuclear inclusion bodies were observed microscopically in the hepatocytes. A herpesvirus was isolated in various primary chicken and duck embryo cell cultures. This virus was not neutralized by antiserum to DVE virus. In experimental transmission studies, the virus caused 100% mortality in adult domestic geese, 50% mortality in 1-day-old commercial ducklings, and 25% mortality in 4–6-week-old commercial ducklings (38). However, Gough and Hansen (22) reported that Pekin ducks are not susceptible to GHV.

In cross-protection studies, Gough and Hansen (22) showed that mortality in DVE-vaccinated geese, Muscovy ducks, and Pekin ducks challenged with GHV was 100, 50 and 0%, respectively. Conversely, in geese, Muscovy ducks and Pekin ducks immunized with inactivated GHV, 100% mortality was recorded in the geese and Muscovy ducks and 80% in the Pekin ducks following challenge with DVE virus. Using cross-neutralization tests in cell cultures, they compared GHV with 5 other avian herpesviruses—namely, DVE/goose, DVE/Muscovy, infectious laryngotracheitis virus, Pacheco's herpesvirus, and falcon herpesvirus. No significant cross neutralization was reported, confirming that GHV is antigenically distinct from DVE viruses. Using restriction endonuclease analysis, a comparison of the genome of GHV with 3 different strains of DVE virus confirmed that GHV is completely different from the DVE viruses. By PCR using two different sets of DVE-specific primers, the GHV was shown to be different from 3 DVE virus strains.

Adenoviruses

Duck adenovirus 1, also known as group III avian adenovirus, egg drop syndrome-1976 virus, avian adenovirus EDS, and egg drop syndrome virus (NCBI & ICTV database), is a member of the genus *Atadenovirus s*pecies duck adenovirus A. Ducks and geese are assumed to be the natural hosts of this virus, but there is no evidence of disease in waterfowl associated with this virus.

Aviadenoviruses have also been reported isolated from a mallard (46) and a Muscovy duck (11). In Muscovy ducks, mortality occurred, but there were no attempts to reproduce the disease with the isolated adenovirus tentatively named duck adenovirus 2.

Unclassified Adenoviruses

Hollmen *et al.* 2003 (32) reported the isolation of an adenovirus from long-tailed ducks (*Clangula hyemalis)* collected during a die-off in the Beaufort Sea off the north coast of Alaska. The authors reproduced the disease experimentally in long-tailed ducks inoculated with the isolated virus; no mortality was recorded but clinical signs included gastrointestinal disease including watery feces and blood in the feces. Challenged ducks seroconverted. The virus could not be neutralized by reference antisera to group I, II or III avian adenoviruses and so may represent a new serotype.

Hollmen *et al.* 2003 (33) also reported on an adenovirus associated with impaction of the posterior small intestine with mucosal necrosis and the cause of death in 10 male common eider ducks (*Somateria mollissima*) in the northern Baltic Sea near Finland. They isolated adenovirus from cloacal swabs from 6 of the birds. The adenovirus could not be neutralized with reference antisera to group I, II or III avian adenoviruses. The virus caused clinical signs of illness and gastrointestinal pathology in an experimentally infected mallard duckling.

Cheng *et al.* 2001 (17) reported attempts to characterize a virus causing enteritis in goslings in China. They identified an adenovirus, and named the virus new gosling viral enteritis virus (NGVEV).

For more information, see Chapter 9, "Adenovirus Infections."

Circovirus-like Infection of Ducks and Geese

Soike *et al*. (67) first reported in 1999 the presence of circovirus-like particles, approximately 15 nm in diameter, by negative stain EM in bursal, splenic, and thymic tissues from a flock of Czech hybrid geese with a history of increased losses and runting. Since then a virus has been isolated from two female mulards showing characteristic signs of circovirus infection (66). This duck circovirus (DuCV)has been shown to be closely related phylogenetically to goose circovirus (GoCV), but is still distinct (27).

Avian circovirus infections which occur in the first months of life are characterized by developmental and/or feathering disorders. The virus invades the lymphoid tissues and leads to immunosuppression, growth retardation and an increased probability of secondary infections, such as *Reimerella anatipestifer* and *Aspergillus* sp. In geese the only apparent gross lesions in 2-week-old and 9-week-old birds were a cloudiness of the air sacs (67). Histopathological changes were evident in the lymphoreticular tissue; lymphocytic depletion and histocytosis were most apparent in the bursa of Fabricius. Basophilic globular inclusions were found in the cytoplasm of medullar and cortical bursal follicular cells and bursal epithelial cells. Ultrastructural examination of these inclusions revealed paracrystalline or multilayered arrays or randomly arranged complexes of isometric viral particles approximately 14 nm in diameter. In naturally infected commercial Muscovy, mule and Pekin ducks, and white Roman geese in Taiwan, the mainly 4–6 week old birds had clinical signs of loss of wing and body feather, necrosis of feather follicles and stunted growth. Grossly the most common lesion was polyseroritis in particular in the pericardial cavity and over the surface of the liver; a cheese like mass within the air sacs was often observed (15).

Smyth *et al.* 2005 (64) investigated a circovirus infection in geese by *in situ* hybridization using a GoCV DNA probe, which showed that circovirus DNA could be demonstrated in the bursa of Fabricius, spleen, thymus, bone marrow, liver, kidney, lung and heart, indicating that the infection can be multisystemic.

Diagnostic tests for GoCV and DuCV are mostly PCR based. Ball *et al.* 2004 (2) reported a PCR and dot blot hybridization test to diagnose GoCV in Hungarian geese. Fringuelli *et al.* 2005 (19) described the development of both conventional PCR and real time PCR to diagnose DuCV. Chen *et al.* 2006 (15) reported a PCR that could detect and differentiate duck and goose circovirus infections.

Glavits *et al.* 2005 (20) investigated an outbreak of West Nile fever in a goose flock in Hungary; they found histologically in addition to the changes attributable to WNV changes more characteristic of a circovirus. Both virus infections were diagnosed by RT-PCR and PCR, respectively, but neither virus was isolated.

A duck circovirus (DuCV) was detected by PCR in bursal and thymic samples from Pekin ducks from New York. The birds exhibited bursal and thymic atrophy, as well as arthritis caused by *Staphylococcus aureus* (3). This is the first report involving Pekin ducks.

Miscellaneous Viral Infections

Tsai *et al.* (75) reported that 77.3% of 611 ducks and 70.9% of 542 geese in Taiwan were positive for antibodies to Japanese encephalitis virus.

Smyth and McNulty (65) reported on a transmissible disease of the bursa of Fabricius in ducks but could not identify a causative agent.

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Duck Hepatitis

P. R. Woolcock

Introduction

Duck hepatitis (DH) is a highly fatal, rapidly spreading viral infection of young ducklings characterized primarily by hepatitis. It can be caused by any of three different viruses, namely duck hepatitis virus (DHV) types 1, 2, and 3. DHV types 2 and 3 were first recognized as separate entities because they induced hepatitis in DHV type 1-immune ducklings. Duck hepatitis is of economic importance to all duck-growing farms because of the high potential mortality if not controlled. The three virus types are not known to have any public health significance. For other reviews of DHV types 1 and 3 see Calnek (15) and for DHV type 2 see Gough and Stuart (48).

In addition to the three viruses that are etiologically associated with liver disease in ducks, a member of the hepadnavirus group

(hepatitis B viruses) is also found in wild and domestic ducks. Although the duck hepatitis B virus is not known to cause disease or lesions in ducks, it is briefly described in a separate section at the end of this chapter.

Duck Hepatitis Type 1

History and Distribution

An acute disease of ducklings, characterized by enlarged livers mottled with hemorrhages, was observed in 1945 by Levine and Hofstad (73). The disease affected ducklings during the 1st wk of age, and death was rapid after signs were observed. While the disease could be transmitted in ducklings, no agent was isolated. During the spring of 1949, Levine and Fabricant (72) studied a highly fatal disease, which is now known as DH type 1, in young white Pekin ducks on Long Island, New York. The disease spread rapidly; before the summer was over, practically all 70-odd duck farms in the area had suffered losses. At first, ducks 2–3 wk old were succumbing. On severely affected farms, mortalities up to 95% were not uncommon in some broods. Successive lots of ducks almost invariably became infected. Later, occasional broods would escape with little mortality. It is estimated that 15% of the total number of ducklings started for that year died from the disease—a total of 750,000 birds. The disease has also been diagnosed in other duck-raising areas of the United States. Duck hepatitis type 1 is worldwide in distribution (107); the most recent reports of new isolations include China (50) and Korea (90).

Etiology

DHV type 1 was first isolated in chicken embryos by Levine and Fabricant (72). No serologic relationship was demonstrated between this virus and that causing duck plague (duck virus enteritis); likewise, no neutralization of DHV type 1 occurred when tested with convalescent serum from cases of human and canine virus hepatitis (28). DHV type 1 contains RNA and has been classified as a picornavirus (106). It bears no relationship to the hepadnavirus infection caused by duck hepatitis B virus (DHBV) as described by Mason *et al.* (82). Duck hepatitis B virus has been found in domestic ducks in China and the United States.

Morphology

DHV type 1 has been estimated to be 20–40 nm in size (97). Richter *et al.* (98) observed 30 nm particles in thin liver sections by electron microscopy (EM). Tauraso *et al.* (106) confirmed the size to be less than 50 nm by filtration studies.

Biologic Properties

Fitzgerald and Hanson (32) were unable to demonstrate hemagglutination of chicken, duck, sheep, horse, guinea pig, mouse, snake, swine, and rabbit red blood cells (RBCs) by cell culture-grown DHV type 1.

Cell cultures infected with DHV type 1 failed to hemadsorb green and rhesus monkey, hamster, mouse, rat, rabbit, guinea pig, human O, goose, duck, and day-old chicken RBCs. High-titered virus suspensions would not hemagglutinate RBCs of the same species when tested at a pH range of 6.8–7.4 and at temperatures of 4, 24, and 37°C (106).

Resistance to Chemical and Physical Agents

DHV type 1 is resistant to ether and chloroform, relatively heat stable, and capable of survival for long periods under usual environmental conditions.

DHV type 1 resisted treatment with ether or fluorocarbon (91), chloroform, pH 3 and trypsin (106), and 30% methanol or ammonium sulfate (53). Using cell culture-grown virus, Davis (20) reported that DHV type 1 resisted pH 3 for 9 hr, but longer exposure (48 hr) reduced virus titer. The virus was not inactivated by 2% lysol or 0.1% formalin (6); 15% creolin, naphthalysol, or xylonaphtha; or 20% anhydrous sodium carbonate (92). Complete inactivation was reported with 1% formaldehyde or 2% caustic soda within 2 hr at 15–20°C, 2% calcium hypochlorite within 3 hr at 15–20°C (92), and 3% chloramine in 5 hr or 0.2% formalin in 2 hr (27). Haider (51) reported complete virus inactivation with 5% phenol, undiluted Wescodyne (an inorganic iodine solution), and undiluted Clorox (sodium hypochlorite solution).

Heating the virus at 50°C for 1 hr had no effect on virus titer (106). Most of the virus was inactivated at 56°C after 30 min (53). Asplin (6) reported, however, that it would survive at 56°C for 60 min but not at 62°C for 30 min. Dvorakova and Kozusnik (27) reported that 23 hr were required for complete inactivation at 56°C. DHV type 1 survived for 21 days at 37°C (91). Heat stability was unaffected by 1 M divalent cation (Mg^{2+})(110). Davis (20), using cell culture-grown type 1 virus, showed that it had a half-life of 48 min at 50°C, but that the presence of molar NaCl, Na_2SO_4, $MgCl_2$, or $MgSO_4$ protected the virus from inactivation at that temperature.

Under more natural environmental conditions, the virus survived at least 10 wk in uncleaned infected brooders and for longer than 37 days in moist feces stored in a cool shed (6). At 4°C, the virus survived over 2 yr (6), (27) and at –20°C for as long as 9 yr (53).

Variability

Viruses differing, or serologically distinct, from DHV type 1 have been recognized as causes of hepatitis in ducklings and have been reported from India (95) and Egypt (104). The Indian isolate is known to be distinct from DHV type l, but its relationship to the other DHV types is unknown.

A variant strain of DHV type 1, named DHV type 1a, has been described by Sandhu *et al.* (102). The origin of the virus is unknown, but all known isolates can be traced back to a single location. With the use of cross-neutralization tests in embryonating chicken eggs, they showed, with somewhat variable results, a partial cross-reaction between types 1 and 1a. They also showed partial cross-protection in passively immunized ducklings challenged with each of the viruses. Woolcock (see (124) had also reported differences between these two viruses in plaque-reduction assays in duck embryo kidney cells. Both DHV type 1 and DHV type 1a are serologically distinct from DHV type 3.

Laboratory Host Systems

Embryos

Levine and Fabricant (72) were the first to propagate the virus in the allantoic sac of 9-day-old chicken embryos. From 10 to 60% of the embryos died by the 5th or 6th day and were stunted or edematous (Fig. 13.1A). Hwang and Dougherty (63) passaged a DHV type 1 strain as two lines in 10-day-old chicken embryos. The serially passaged lines became nonpathogenic for newly hatched ducklings at the 20th and 26th transfers. The virus titer in chicken embryos was 1–3 $\log_{10}$ lower than when grown in ducklings.

Hwang (55) developed a chicken embryo lethal strain of DHV type 1 by serial embryo passages. Using a homogenate of dead embryos and chorioallantoic membrane (CAM) in embryonic fluid, mortality reached 100% at the 63rd passage. More consistent results were obtained when 5- to 7-day-old embryos were inoculated via the yolk sac.

Toth (108) found titers of 80th passage-adapted virus to be highest at about 53 hr postinoculation (PI): embryo, $10^{7.50}$; CAM $10^{5.79}$; and amnioallantoic fluid $10^{3.62}$. A high-titer live vaccine could be harvested from all these parts 53–69 hr PI. Essentially similar results were reported by Pan (87).

Mason *et al.* (81) noted a somewhat higher titer of attenuated DHV type 1 in chicken embryos that reached a peak (10^8) in 48 hr. The latent period was between 6 and 24 hr.

Goose embryos were found to be susceptible to the virus, and embryo deaths occurred 2–3 days after allantoic inoculation (4).

Cell Cultures

Various attempts to grow and assay DHV type 1 in cell cultures of duck and chicken embryo origin have been described (32), (34), (57), (65), (81), (91). Maiboroda (76) followed development of DHV type 1 in monolayers of duck kidney cells with a direct fluorescent antibody (FA) technique. Fluorescence was observed after 8 hr, reached a maximum after 2–4 days, and was confined to the cytoplasm. Cytopathic effects (CPE) (rounding of cells) and maximum virus titers occurred after 2 days. Maiboroda and Kontrimavichus (77) produced growth and CPE in goose embryo kidney cells. Kurilenko and Strelnikov (71) reported similar results in piglet kidney cell culture. Davis and Woolcock (23) showed that attenuated DHV type 1 grew in embryo cell cultures of goose, turkey, quail, pheasant, guinea fowl, and chicken origin, while virulent virus strains grew to varying degrees in only guinea fowl, quail, and turkey embryo cells. Golubnichi *et al.* (42) reported successful growth and a high level of cytopathogenicity in duck embryo fibroblasts inoculated with chick embryo-adapted DHV type 1. They recommended this procedure for vaccine production and virus neutralization (VN) tests.

Woolcock *et al.* (121) described a plaque assay for attenuated DHV type 1 in primary monolayers of duck embryo kidney (DEK) cells. The concentration of fetal calf serum in the overlay medium affected plaque size. Subsequently, Chalmers and Woolcock (17) demonstrated that several mammalian sera had an inhibitory effect on the virus, which was nonspecific and only occurred when the serum was in direct contact with the virus. The virus-inhibitory substance in fetal calf serum appeared to be present in the albumin fraction. There was no or minimal inhibitory effect in sera from ducks or chickens. Woolcock (116) reported plaque assays for both virulent and attenuated DHV type 1 in primary duck embryo liver (DEL) cells and compared the results of *in vitro* assays with those obtained *in ovo* and *in vivo*. Kaleta (66) described a microneutralization assay using attenuated DHV type 1 in primary DEK cells; Woolcock (117) modified this assay to monitor immune responses to vaccines.

Pathogenicity

Asplin (5) and Reuss (96) reported loss of pathogenicity of DHV type 1 for ducklings after chicken embryo passages. Hwang (55) found one virus strain to have lost its pathogenicity for ducklings after 20 or more passages in chicken embryos. He also found that the same strain had lost its pathogenicity for ducklings after the sixth passage in duck embryo fibroblasts, but the virus retained its pathogenicity for chicken embryos (56).

Hwang and Dougherty (63) reported that chicken embryo-passaged strains, while nonpathogenic for ducklings, did multiply in the tissues but at a lower titer than field strains. Field strains were found in fairly high concentrations in duckling brain; chicken embryo-passaged strains could not be detected or were present in low concentrations in the brain.

A similar attenuation of pathogenicity has been reported when DHV type 1 was passaged in duck embryos (11). Embryo passage-attenuated DHV type 1 strains are still capable of causing very mild and transitory histologic changes after inoculation (101), (105), and reversion to virulence occurs after backpassage in young ducklings (122), (123).

When Kapp *et al.* (67) encountered heavy losses from DH type 1 in several flocks of ducklings 3–4 wk old, they suspected inadequate rations as contributory causes. This was borne out experimentally when eight of nine 3-wk-old ducks that had been fed the farm ration died after virus exposure. No mortality occurred in controls on normal feed. It was concluded that faulty diet had impaired liver function, which predisposed ducklings to hepatitis at an unusually advanced age.

Friend and Trainer (35, 37, 38) fed low levels of polychlorinated biphenyl, DDT, and dieldrin to mallard ducklings for 10 days; 5 days later, birds were infected with DHV type 1. Birds receiving toxic substances had significantly higher mortality than controls not previously fed the chemicals. It appears that inadequate diet or ingestion of toxic substances exacerbates pathogenic effects of the virus.

Lu *et al.* (74) reported an outbreak of infectious-bill-atrophy syndrome in ducklings in Taiwan, which they believe was caused by a parvovirus infection in association with DHV. The exact role of DHV in this syndrome was not clearly defined.

Sandhu *et al.* (102) reported that the pathologic responses to DHV types 1 and 1a were similar.

Pathogenesis and Epidemiology

Natural and Experimental Hosts

In naturally occurring outbreaks, DH type 1 occurred only in young ducklings. Adult breeders on infected premises did not be-

come clinically ill and continued in full production. Field observations indicated that chickens and turkeys were resistant. Rahn (94), however, found that day-old and wk-old poults exposed to DHV type 1 developed signs, lesions, and neutralizing antibody. Poults, after either oral or intraperitoneal exposure, had mottled livers and enlarged gall bladders and spleens. DHV type 1 was isolated from livers up to 17 days after oral exposure of day-old poults. Schoop *et al.* (103) and Reuss (97) failed to infect chickens experimentally. Reuss could not transmit the disease to rabbits, guinea pigs, white mice, or dogs. Asplin (6) reported that young chickens can contract an inapparent infection and pass it on through contact with other chicks. Experimental infections in goslings (4) and mallard ducklings (36) have been reported. In experimentally exposed birds, no mortality occurred in chicks, Muscovy ducklings, or pigeon squabs; low mortality occurred in young turkeys and quail, while high mortality occurred in young pheasants, geese, and guinea fowl. All exposed birds became infected with DHV type 1 (61).

Transmission, Carriers, and Vectors

Under field conditions, DH type 1 spreads rapidly to all susceptible ducklings in the flock. Although high mortality and rapid spread of the disease on farms indicate extreme contagiousness, occasional exceptions have been observed. In one pen, 65% of the ducks died, while in an adjoining pen separated only by a 14-in. curb, mortality was negligible.

The first efforts to transmit the disease to small groups of three or four caged ducklings by injection and feeding of egg-propagated virus were not successful. In another experiment, with tissues from a naturally occurring outbreak, some ducklings became infected. Transmission was most easily accomplished by intramuscular (IM) injection and feeding egg-propagated virus and infected organs to larger groups of ducklings (10–20) kept on litter under a hover. The incubation period was 24 hr in most experiments, and nearly all deaths took place by the 4th day. Uninoculated ducklings placed in the same pens with inoculated birds contracted the disease and died somewhat later than injected ducks.

Egg transmission presumably does not take place. Newly hatched ducklings produced by breeders on infected premises remained well when taken where no ducks were being kept. Asplin (5) confirmed this finding.

Priz (93) found that aerosol infection of ducklings with Yagotinski strain of DHV type 1 was lethal.

Hanson and Tripathy (54) reported successful infection with attenuated DHV type 1 by the oral route, although Toth and Norcross (111) suggested that in this case, the portal of entry was really the pharynx or upper respiratory tract, since the virus administered in a capsule failed to produce infection.

Recovered ducks may excrete virus in feces for up to 8 wk PI (97). Asplin (6) reported that there is strong field evidence to incriminate wild birds as mechanical carriers of the virus over short distances. He also suggested the possibility that an unknown host acting as a healthy carrier might be responsible for new outbreaks at great distances. However, Asplin (8) found no serologic evidence of DHV type 1 in VN tests of sera from 520 wild aquatic fowl of six species. These negative results were fortified by failure of Ulbrich (113) to find VN antibodies in 36 wild ducks (four species) taken from ponds where DH type 1 had occurred in domestic ducks. In addition, all of 153 wild duck embryonated eggs from an infected area were susceptible to experimental infection.

Of possible significance in the epizootiology of the disease is the report of Demakov *et al.* (25) indicating that brown rats (*Rattus norvegicus*) could act as a reservoir host of DHV type 1. Ingested virus remained alive in the body up to 35 days and the virus was excreted 18–22 days PI. Serum antibodies were also present 12–24 days PI.

Vectors are not known to be a factor in transmission of DH type 1.

Signs

Onset and spread of DH type 1 are very rapid, with practically all mortality occurring within 3–4 days. Affected ducklings at first fail to keep up with the brood. Within a short time, they stop moving and squat down with eyes partially closed. Birds fall on their sides, kick spasmodically with both legs, and die with heads drawn back (Fig. 13.1B). Death occurs within an hour or so after signs are noted. During the height of severe outbreaks, the rapidity with which ducklings die is astonishing.

Farmer *et al.* (29, 30) described duck fatty kidney syndrome and focal pancreatic necrosis, which were considered to be aspects of DH type 1. Between 1978 and 1983, losses up to 30% were recorded on certain duck-rearing farms in the United Kingdom. Two age groups, 1–2 wk and 4–6 wk old, were affected despite routine vaccination with type 1 vaccine. Gross lesions were characterized by pale swollen livers and kidneys, and swollen mottled spleens. Histologic evidence was indicative of DH type 1. Despite vaccination with attenuated DHV type 1, and the age of the older group of birds, the authors suggested that this syndrome was a manifestation of DH type 1. They have acknowledged, however, that DHV type 2, which was subsequently diagnosed in East Anglia (45, 46), may have played a role in this syndrome.

Morbidity and Mortality

Morbidity is 100% and mortality is variable in young ducklings infected with DHV type 1. In some broods less than 1 wk old, mortality may reach 95%. In ducklings 1–3 wk of age, mortality may be 50% or less. In ducklings 4–5 wk of age, morbidity and mortality are low or negligible.

Gross Lesions

Principal lesions due to DHV type 1 are found in the liver, which is enlarged and contains punctate or ecchymotic hemorrhages (Fig. 13.1C). Frequent reddish discoloration or mottling of the liver surface is seen. The spleen is sometimes enlarged and mottled. In numerous cases, the kidneys are swollen and renal blood vessels congested.

Histopathology

Microscopic changes in uncomplicated, experimentally induced DHV type 1 infections have been studied (28). Primary changes

in the acute disease consisted of necrosis of hepatic cells (Fig. 13.1D); survivors with more chronic lesions showed widespread bile duct hyperplasia (Fig. 13.1E). Varying degrees of inflammatory cell response and hemorrhage occurred. Regeneration of liver parenchyma was observed in ducklings that did not die. Ten-day-old chicken embryos inoculated with DHV type 1 were killed and examined histologically at 12-hr intervals for periods up to 10 days PI (33). Histologic changes included proliferation of granulocytes in various organs, focal necrosis of the liver, bile duct hyperplasia, and subcutaneous edema. Inclusion bodies were not found. Six-day-old ducklings were infected intranasally and intramuscularly with DHV type 1 and killed 14–24 hr later. Their livers were examined by EM. One hr after infection, there was an occasional breakup of glycogen within the liver cells. Spherical particles 100–300 nm in diameter and of unknown origin were visible. Changes seen in peracute cases were degenerative, and there was extensive cell necrosis after 24 hr. Virus-like particles were detected at 1 hr and 18–20 hr PI (1).

Adamiker (2) examined spleen and muscle of ducks infected with DHV type 1 by EM. The spleen showed regressive changes from the 6th hr PI and became necrotic by the 24th hr. There were degenerative changes in the nuclei of plasma cells that may have been caused by the virus. Virus particles were not identified. Only slight changes were seen in muscles.

Biochemical Effects

Ahmed *et al.* (3) reported that in clinical cases of DH type 1, there were lower serum levels of total protein and albumen and elevated levels of alkaline phosphatase, glutamic pyruvic transaminase (GPT), bilirubin, and creatinine. Mennella and Mandelli (83) noted that serum levels of GPT and glutamic oxaloacetic transaminase were increased in relation to severity of infection. Buynitzky *et al.* (12, 13) indicated that even in clinically inapparent DHV type 1 infection in mallards, liver enzyme patterns were altered, with a consequent alteration in metabolism of DDT. This may partially explain the interrelationship between chlorinated hydrocarbons and DH type 1 (35, 37, 38).

Immunity

Recovery from DH type 1 results in solid immunity and VN antibodies in the serum. Active immunity can be induced in adult ducks by injection of certain strains of virus (6). Some strains require repeated injections to obtain high levels of antibody (96). Passive immunity can be conferred to ducklings by injection of serum from recovered or immunized birds. Passive antibodies may also be transferred through yolk to hatched ducklings to protect them. Malinovskaya (79), investigating the antibody response to DHV type 1 vaccine in breeder ducks and 7-day-old ducklings, showed by a passive hemagglutination (HA) test that duck serum contained more 7S antibodies (cysteine sensitive) than 19S antibodies (cysteine resistant). Decline of the 7S antibodies halved the hemagglutination-inhibition titers in 43% of serum samples taken 3–7 days PI. In ducklings experimentally infected between 3 and 21 days of age, the main type of antibody response was 19S during the ensuing 20 days; but in ducklings infected at 30 days of age, 19S antibody was formed first, but 7S antibody began to appear after 15 days. Davis and Hannant (22) reported that VN antibody was present 4 days postvaccination of 2-day-old ducklings. The antibodies were shown to be in the macroglobulin and 7S fractions by Sephadex G200 chromatography and had γ or $ß_2$ mobility by immunoelectrophoresis.

Diagnosis

Virus Isolation and Identification

The presence of DHV type I may be confirmed by one or more of the following procedures Woolcock (119, 120):

- Inoculation, subcutaneously (SC) or intramuscularly (IM), of the isolate into 1-to-7-day-old DHV type I-susceptible ducklings. The characteristic clinical disease should follow, with deaths often occurring within 24 hours. Ducklings should show the gross pathology attributable to DHV type I. The virus should be reisolated from the livers to confirm the diagnosis.
- Inoculation of serial dilutions of the liver homogenate into the allantoic sacs of embryonated duck eggs (aged 10–14 days) from a DHV type I-free flock, or chicken eggs (aged 8–10 days). DHV type I-infected duck embryos should die within 24–72 hours; chicken embryos are more variable and erratic in their response and usually take 5–8 days to die. The allantoic fluid is opalescent or a pale greenish-yellow. Gross pathological changes in the embryos include stunting and subcutaneous hemorrhages over the whole body, with edema, particularly of the abdominal and hind limb regions. The embryo livers may be swollen, red and yellowish in color, and show necrotic foci. The liver lesions and embryo stunting become more apparent in embryos that take longer to die.
- Inoculation of primary cultures of duck embryo liver cells (116). Serial dilutions of the liver homogenate containing DHV type I cause a cytopathic effect (CPE) that is characterized by cell rounding and necrosis. When overlaid with a maintenance medium containing 1% agarose (wt/vol), the CPE gives rise to plaques approximately 1 mm in diameter.

A rapid and accurate diagnosis of DH type 1 can be made using the direct FA technique on livers of naturally occurring cases or inoculated duck embryos (76), (114).

Virus isolation and identification procedures have been previously reviewed (51, 110).

Serology

Serologic tests have not been useful in diagnosing acute outbreaks of DHV type 1. However, from the time the virus was first recognized (72), the VN test has been used for other purposes: virus identification, titration of serologic response to vaccination, and epidemiologic surveys.

Hwang (58) described an accurate, reproducible DHV type 1 neutralization test in chicken embryos. Modifications of this procedure were described (47, 111). Haider (51) described several modified VN tests in duck embryos or ducklings, and Golubnichi *et al.* (42) described a VN test with virus adapted to tissue culture. Malinovskaya (78) reported that a passive HA test was more

sensitive than the VN test. Ivashhenko (64) examined the use of an indirect HA test for the diagnosis of DHV type 1 and showed a 90% correspondence between indirect HA and VN results.

Murty and Hanson (84) described the use of an agar gel diffusion precipitin (AGDP) test for identification of DHV type 1. Later studies by Wachendorfer (115) and Toth and Norcross (111) claimed that reactions seen by Murty and Hanson were not specific or related to DHV type 1 or antibodies. Zhao *et al.* (125) compared enzyme-linked immunosorbent assay (ELISA), VN, and AGDP for the detection of DHV type 1 antibodies in duck sera. In a report containing several errors, they claimed ELISA and VN to be comparable in sensitivity, but they did not quantitate their VN test results; AGDP was reported to be considerably less sensitive, but exactly what was being measured must be questioned in the light of other reports (111, 115).

Woolcock *et al.* (121) first described a plaque-reduction test for VN antibody. This assay was considerably more sensitive than VN assays in eggs. Chalmers and Woolcock (17) showed that sera collected from 16 uninfected ducks had 50% plaque reduction titers (VN_{50}) ranging between 1:12 and 1:250 with an average of 1:59. They suggested that the maximum VN_{50} titer for negative control serum should be taken as 1:250. Woolcock (116) reported a plaque reduction assay in DEL cells and showed that type 1 virus was only neutralized by type 1 antiserum and not by antisera to type 2 or type 3. He also reported that a VN_{50} of 1:64 in embryonated chicken eggs was equivalent to a VN_{50} in excess of 1:3200 in DEK cells. Kaleta (66) described a microneutralization assay for DHV type 1 in DEK cells. He claimed the assay to be more practical, rapid, and economical than alternative tests but considered the plaque reduction assay more sensitive. Woolcock (117) adapted this microneutralization assay to monitor the VN antibody responses of ducks to vaccines in field and laboratory trials.

Differential Diagnosis

The sudden onset, rapid spread, and acute course of the disease caused by DHV type 1 are characteristic. Hemorrhagic lesions in livers of ducklings up to 3 wk of age are practically pathognomonic. Occurrence of similar disease outbreaks, caused by serologic variants of type 1 virus or by DHV type 2 and DHV type 3, offers the main problem in differential diagnosis.

Chalmers *et al.* (16) reported an outbreak of DHV type 1 associated with *Chlamydia psittaci* in 4- to 6-wk-old birds and suggested a possible synergistic effect as the cause of the persistent 15% mortalities recorded. Gough and Wallis (49) reported DHV type 1 associated with influenza virus in 2- to 5-wk-old mallard ducks reared on a game farm. The DHV type 1 isolated was of low virulence, and it is suggested that the influenza virus may have exacerbated the hepatitis infection.

Other potential causes of acute mortality in ducklings include salmonellosis and aflatoxicosis. The latter disease may cause ataxia, convulsions, and opisthotonos as well as microscopic lesions of bile duct hyperplasia suggestive of DH but does not cause the same characteristic liver hemorrhages. None of the other common lethal diseases of ducks occur frequently in this young age group.

Treatment

As soon as the cause and nature of DH type 1 were recognized by Levine and Fabricant (72), it became apparent that ducklings might be protected by administration of serum from immune ducks. This procedure proved to be highly successful in laboratory experiments and in the field. For many years, the Duck Research Laboratory at Eastport, Long Island, kept a bank of antiserum processed from blood collected at the time of slaughter from recovered birds. Intramuscular injection of 0.5 ml DHV type 1 antiserum into each duckling of a brood at the time of the first deaths in an outbreak was an effective control method.

Rispens (100) suggested passive immunization by injection of yolk from eggs produced by hyperimmune breeder ducks. At the Duck Research Laboratory, Long Island, New York this procedure was modified by substituting yolk from eggs produced by specific-pathogen-free chickens hyperimmunized with DHV type 1.

Prevention and Control

Management Procedures

Duck hepatitis type 1 can be prevented by strict isolation, particularly during the first 4–5 wk. In areas where the disease is prevalent, however, it is very difficult to obtain the necessary degree of isolation.

Panikar (88) and Kaszanyitzky and Tanyi (68) demonstrated the feasibility of eradicating DH type 1 in selected areas where isolation can be achieved. In both studies, vaccination of breeder ducks was used as part of the program.

Immunization

Resistance against DH type 1 may be conferred to ducklings by three methods: injection of immune serum or yolk as described under Treatment; immunization of breeding stock to ensure high levels of passively transferred antibody in the hatched ducklings; and direct active immunization of ducklings with live avirulent strains of DHV type 1.

Attenuated DHV type 1 strains suitable for vaccine use have been produced by passage in chicken embryos (5, 39, 40, 42, 62, 103) or duck embryos (100). Up to this time, various strains of chicken embryo-passaged DHV type 1 have been used most frequently as vaccines.

Davis (21) reported that triple plaque-purified strains of DHV type 1 vaccines could revert to virulence as readily as noncloned virus. This finding has been confirmed with various egg-passage levels of DHV type 1 (118). Davis suggested rapid passage as a method to increase genetic stability.

Breeders. Asplin (5) developed a chicken embryo-attenuated strain of virus for immunizing breeders. The method was to inoculate 0.5 mL undiluted egg-propagated virus IM 2–4 wk before collecting hatching eggs. Reuss (96) found it necessary, with the strain of virus he used, to make repeated injections in breeders to obtain sufficient antibody levels to protect hatched ducklings against challenge with virulent virus. The

optimum age, dosage, route of inoculation, strain of virus, and interval between initial and subsequent vaccinations are not known (6).

Rispens (100) recommended two doses of attenuated virus vaccine administered to breeders at least 6 wk apart; passive immunity was transmitted to progeny for about 9 mo after the second vaccination.

Hwang (59), Rinaldi *et al.* (99), Nikitin and Panikar (85), and Doroshko and Bezrukavaya (26) all confirmed that two or three doses of attenuated virus vaccine were necessary to secure satisfactory levels of protection of progeny. Bezrukavaya (11) secured effective protection by vaccination of breeders with duck embryo-attenuated vaccine. Demakov *et al.* (24) reported that effectiveness of the chicken embryo-attenuated strain was improved by aluminum hydroxide adsorption and a saponin adjuvant. Malinovskaya (80) looked at the effect of chemical stimulators on postvaccinal immunity against DHV type 1 by assaying for passive HA and VN antibodies. Dibazol had no effect, but saponin, methyluracil, and ascorbic acid promoted an accelerated antibody response, which was particularly pronounced 15 days postvaccination.

Golubnichi and Malinovskaya (41) monitored the immune response, following up to three immunizations over a 3-mo period, by assaying HA and VN antibody. The antibody titers in sera of laying ducks necessary to protect their offspring was 1:64 for HA and 1:32 for VN antibodies.

The application of inactivated vaccines also has been investigated. Gough and Spackman (47) reported that effective levels of duckling protection can be secured by administering three doses of inactivated DHV type 1 oil-emulsion vaccine. These workers also reported that live DHV type 1 vaccine at 2–3 days of age, followed by inactivated vaccine at 22 wk, produced significantly higher VN antibody levels than did three doses of inactivated vaccine. Finally, they reported that inactivated vaccine prepared from virus grown in duck eggs gave a better antibody response than virus grown in chicken eggs. Woolcock (117) investigating the use of inactivated DHV type I vaccines in breeder ducks, showed that in order to ensure an adequate immune response to the inactivated virus, it was necessary to prime the ducks with DH type 1-modified live virus (MLV). He showed that ducks primed with MLV at 12 wk of age, and boosted with inactivated DHV type 1 vaccine at 18 wk, developed VN antibody titers 16-fold higher than those in ducks that received only the MLV priming. This level of immunity was sufficient to protect ducklings hatched through a complete laying cycle (8 mo), as demonstrated by challenging progeny with virulent DHV type 1. Inactivated vaccines prepared from MLV grown in embryos or from virulent type 1 virus grown in ducklings were both equally effective. Woolcock (117) also investigated various immunization schedules, using inactivated virus alone, and monitored the VN antibody response of individual ducks in a microtiter assay using primary DEL cells. Only 7 (11%) of 63 ducks developed titers of 6 $\log_2$ or greater, which was considered the minimum protective level, and these responses were only in birds given multiple inoculations of inactivated vaccine.

Ducklings. Asplin (5) used his chicken embryo-attenuated strain of DHV type 1 to vaccinate ducklings by the foot web-stab method. Reuss (96) also reported successful immunization experiments with an attenuated strain.

Newly hatched ducklings injected IM with an attenuated DHV type 1 developed resistance in 3 days (60). Oral exposure required up to 6 days for protection to occur. There was evidence that vaccination would be of benefit even at the start of an outbreak.

Lyophilized, attenuated strains of DHV type 1 induced a considerable degree of protection in day-old ducklings inoculated by the IM, intranasal, or foot-web route (126). Crighton and Woolcock (19) and Gazdzinski (40) also reported successful immunization of ducklings by the (subcutaneous) SC and IM routes, respectively, with chicken embryo-passaged DHV type 1. Golubnichi *et al.* (42) used tissue culture-passaged DHV type 1 for duckling immunization. Effective mass vaccination of ducklings by the aerosol and drinking water routes have been reported (54, 69, 70, 86, 89, 112). Ducklings vaccinated orally at 2–3 days of age did not show an increased immune response when revaccinated at 17 days (9). Balla *et al.* (10) examined administration in the field of DHV type 1 vaccine by SC and drinking water routes. They reported that two doses given in the drinking water at 2–3 days of age were as effective as one dose given orally at 2 days of age.

Balla and Veress (9) examined the antibody response of ducklings of different immune status to SC and oral vaccination with live attenuated DHV type 1. They found that susceptible ducklings and those hatched with maternally derived immunity both responded to vaccination with 1600–9600 EID_{50} given during the first 3 wk of life. The maternally immune birds responded only marginally less. They also showed that a dose of 300–600 EID_{50}/duckling between 2 and 21 days and of 100 EID_{50} at 35 days was sufficient for seroconversion to occur irrespective of the ducklings' immune status. Exposure to an aerosol vaccine for 5–6 min at 2–5 days of age produced a good response in susceptible birds but not in maternally immune ducklings; 30-min exposure to aerosol vaccine gave a good response, which was not boosted by reexposure at 16 days. They did not report any results covering challenge of any of these ducklings with virulent virus. Luff and Hopkins (75) also looked at the effect of maternally derived immunity on vaccination of ducklings with live attenuated DHV type 1. Ducklings were vaccinated when about 12 hr old and were challenged with virulent DHV type 1 at 24 hr, and 3 and 6 days. Their results suggested that partially protective levels of maternally derived antibody did not adversely affect either the speed of onset or the extent of protection afforded by the currently available live vaccine. Unfortunately, findings were limited to the first 6 days of life.

In contrast to the results reported by Luff and Hopkins, field experience has indicated that successful practical duckling vaccination is dependent on the absence of maternal antibodies and is influenced by time and severity of exposure to virulent virus. Vaccination is also less effective when ducklings are exposed to virulent virus early in life, especially in endemic areas and on

heavily infected premises. Judicious application of proper hygiene and sanitation methods could do much to solve this problem.

Duck Hepatitis Type 2

Duck hepatitis type 2, although similar to DH type 1 as a pathologic entity, is caused by an entirely different agent (48). The first report of an outbreak of DH type 2 in ducklings was from Norfolk, England, in 1965 (7). The affected flock had been vaccinated with attenuated DHV type 1. An agent was isolated which was shown, by cross-protection studies in ducklings, to be different from DHV type 1 and was named DHV type 2 (7). The disease disappeared from commercial flocks by 1969 but reappeared in 1983/84 on three farms, again in Norfolk, England (45). Losses in that outbreak varied between 10 and 25% in 3- to 6-wk-old birds, and up to 50% in 6- to 14-day-old birds. Outbreaks on affected farms were often sporadic, affecting some batches of ducks and not others. There are no reports of the disease occurring outside of East Anglia, England, and since the outbreaks in the mid-1980s, there have been no more outbreaks of the disease in that area (44).

DHV type 2 particles have an astrovirus-like morphology and a diameter between 28 and 30 nm as seen by EM (46). Aggregates containing more than 1000 virus particles have been observed in liver suspensions. On this basis, DHV type 2 has been classified as an astrovirus, and it has been suggested that it should be renamed duck astrovirus (43). It has been compared with astrovirus isolates from chickens and turkeys by cross-protection and transmission studies, and found to be antigenically distinct (48). The virus is resistant to chloroform, pH 3.0, trypsin treatment, and heating at 50°C for 60 min. Formaldehyde fumigation and standard disinfection procedures have eliminated the infection from contaminated premises (48).

DHV type 2 replicated in embryonated chicken eggs following several blind passages in the amniotic sac (45). Few embryos died in less than 7 days, but infected embryos appeared stunted and had greenish necrotic livers in which astrovirus-like particles could be demonstrated by EM. Attenuation of pathogenicity occurred after serial passage in chicken embryos (45). Various duck and chicken cell cultures appear to be refractory to infection (45), (116).

Ducks appear to be the only species affected by DHV type 2, and no wildlife reservoirs nor vectors have been detected. All recorded outbreaks have initially involved ducks kept on open fields; therefore, wildfowl, gulls, and other wild birds have been suspected as being vectors (43).

Infection occurs through oral, cloacal, and subcutaneous routes. Deaths occur within 1–4 days, usually within 1–2 hr after the appearance of clinical signs, which include polydypsia with loose droppings, excessive urate excretion, and sometimes convulsions and acute opisthotonos (48). Affected ducks usually die in good condition and both the time of death and the mortality rate (10–50%) depend on the age of the ducks (45). Survivors excrete virus for at least 1 wk after infection (48) and rear normally, with little evidence of retarded growth (45). Mature ducks are refractory to the disease (45).

The target organs for DHV type 2 appear to be liver and kidneys (45). Significantly more virus is present in the liver, which is usually pale pink with multiple, small punctate hemorrhages, often forming confluent bands. The spleen is invariably enlarged and "sago-like" in appearance due to scattered pale foci. Kidneys are often swollen with blood vessels injected and standing out from the pale kidney substance. The alimentary tract is usually devoid of food. Occasionally, small hemorrhages are seen in the intestinal wall and on the heart fat. Microscopic changes in the acute disease are characterized by extensive necrosis of the hepatocyte cytoplasm and bile duct hyperplasia is usually present and widespread.

Gough and Smart (48) found that survivors of DH type 2 were immune to further infection. Detectable antibody levels following infection were shown to be low, using a varying virus-constant serum neutralization test in embryonated chicken eggs (43).

The most reliable diagnostic method for DHV type 2 is EM examination of liver homogenates for the detection of astrovirus-like particles. The virus can only be isolated, with difficulty, following repeated passage in the amniotic sac of embryonated chicken or duck eggs. Inoculation of susceptible ducklings with virus gives a variable response; mortality up to 20% may occur within 2–4 days PI (45, 119, 120).

Inoculation of susceptible ducklings with convalescent serum obtained from DHV type 2-infected ducks has been used successfully to control the disease in the field (48). An experimental live attenuated virus vaccine also protected ducklings from challenge with virulent virus, but this vaccine has never been produced commercially (48).

Duck Hepatitis Type 3

Duck hepatitis type 3 was first reported by Toth (109) who observed hepatitis causing mortality and morbidity in ducklings immune to DHV type 1, on Long Island in the United States. The disease was less severe than DH type 1 and mortality rarely exceeded 30%. Based on differences from both DHV type 1 and DHV type 2, the agent was named DHV type 3 (52). The disease is only known to have occurred in the United States.

Haider and Calnek (52) reported that DHV type 3 contained RNA, based on insensitivity to IUdR, and was resistant to chloroform and pH 3.0 but sensitive to 50°C irrespective of the presence of 1 M $MgCl_2$. Electron microscopy of cultured duck kidney (DK) cells infected with the virus revealed crystalline arrays containing particles about 30 nm in diameter in the cytoplasm. Based on these observations, they suggested that DHV type 3 be classified as a picornavirus, but that it was unrelated to DHV type 1, since no common antigens could be demonstrated in VN and FA tests.

Nine- to 10-day-old duck embryos inoculated onto the CAM were susceptible to DHV type 3 (52). During the first passages embryo deaths were erratic and did not occur until the 8th or 9th day PI, but this was reduced with higher passages. In severely affected embryos, the CAMs were discolored and the surface of the affected areas had a dry crusty or cheesy appearance. Underneath, the CAM was edematous and thickened up to 10

times normal. Embryo lesions included stunting, edema, skin hemorrhages, flaccid appearance, gelatinous fluid accumulations and enlargement of liver, kidneys, and spleen. Attenuation of pathogenicity for ducklings, accompanied by increased pathogenicity for duck embryos, occurred following serial passage in embryonated duck eggs inoculated by the CAM route. Chicken embryos were not susceptible to inoculation with DHV type 3.

Liver and kidney cell cultures of duck embryo or duckling origin were shown to support replication of the virus. This was demonstrated by a direct FA test to show foci of positively infected cells (52). Woolcock (116) reported that the type 3 virus failed to produce plaques in primary DEK and DEL cell monolayers.

DHV type 3 has a low pathogenicity for ducklings experimentally infected, and only ducklings appear to be affected by the virus. Subcutaneous or IM inoculation of liver homogenate from infected ducklings into susceptible day-old ducklings is unreliable. Intravenous inoculation may increase the effectiveness. Mortality and virus yields from liver could be increased if ducklings received two or three doses of cyclophosphamide (2 mg/dose) on days 1–3 and were challenged with virus on the 6th day of age (14).

Ducklings dying from DHV type 3 infection show the typical appearance of type 1 infection, i.e., outstretched legs and opisthotonos (109). Mortality rarely exceeds 30%, but gross pathologic changes are similar to those caused by DHV type 1.

An active immune response to DHV type 3 can be stimulated in adult ducks by inoculation of attenuated virus. This immunity may be passively transferred, via the yolk, to progeny.

DHV type 3 infection may be tentatively identified by inoculation of liver suspension onto the CAM of 10-day-old embryonated duck eggs if embryo lesions and mortality pattern develop as described above (119, 120). Alternatively, virus may be isolated and identified in DK or DEK cultures examined by immunofluorescence 48–72 hr PI, using DHV type 3-specific antiserum. A direct FA test in duckling livers and DEK or DK cells has been described for DHV type 3 (52). A serum neutralization test in embryonated duck eggs is possible. Differential diagnosis is similar to that described for type 1 virus.

Convalescent sera obtained from DHV type 3-infected ducks has been used effectively in the field to control outbreaks. A live attenuated vaccine has been used experimentally in breeder ducks to confer passive immunity to ducklings, but this vaccine has not been available commercially.

Duck Hepatitis B Virus Infection

Duck hepatitis B virus (DHBV) infection is widely distributed in domestic ducks and several species of migratory wild ducks. It is commonly found in the liver and serum. The virus is a small (40 nm diameter) DNA virus belonging to the hepadnavirus group which includes human and woodchuck hepatitis B viruses. In contrast to these mammalian hepadnaviruses, DHBV has not been associated with significant lesions or clinical disease in either chronic congenitally acquired infection or acute experimentally induced infection.

A detailed review of the characteristics of this virus can be found in (31).

Chang *et al.* (18) reported a new avian hepadnavirus infecting snow geese (*Anser caerulescens*). Sequence analysis of the PCR-amplified genome revealed that this virus was distinct from other avian hepadnaviruses.

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Duck Virus Enteritis (Duck Plague)

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Introduction

Definition and Synonyms

Duck virus enteritis (DVE) is an acute, contagious herpesvirus infection of ducks, geese, and swans, characterized by vascular damage, tissue hemorrhages, digestive mucosal eruptions, lesions of lymphoid organs, and degenerative changes in parenchymatous organs. Synonyms for the disease are *duck plague*, *eendenpest* (Dutch), *peste du canard* (French), *Entenpest* (German), and *duck virus enteritis* (90). Although Bos (4) first used the term *duck plague*, it was proposed as the official name by Jansen and Kunst in 1949 (39). Subsequently, *DVE*, based on principal features of the disease and to distinguish it from fowl plague, has become the preferred term.

Public Health Significance

DVE is primarily a disease of waterfowl. No known risk to human health has been reported.

Economic Significance

In duck-producing areas of the world where the disease has been reported, DVE has produced significant economic losses in domestic and wild waterfowl due to mortality, condemnations, and decreased egg production. The first outbreak in the United States in 1967 caused losses in excess of $1 million during a 1-year period for the small, but concentrated, duck industry of Long Island, New York (54).

History

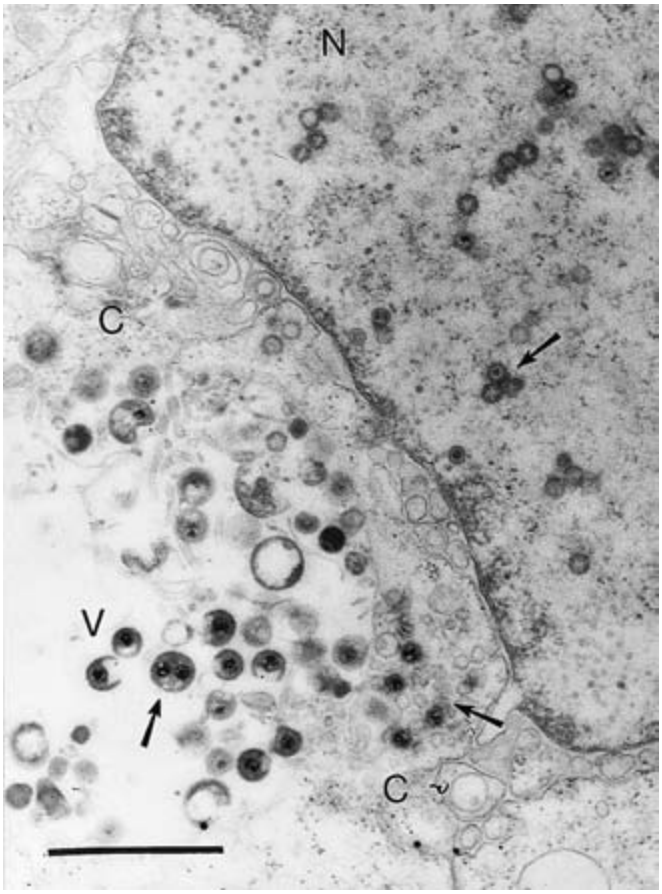
In 1923, Baudet (2) reported an outbreak of an acute, hemorrhagic disease of domestic ducks in the Netherlands. Bacterial cultures were negative, and the disease was experimentally reproduced in domestic ducks by injection of sterile filtered liver suspensions. Although presented as a previously unknown viral infection of ducks not infecting chickens, it was concluded the disease was due to a specific duck-adapted strain of fowl plague (avian influenza) virus. Subsequently, more outbreaks were reported in the Netherlands. DeZeeuw (20) substantiated Baudet's findings and speculated the presence of a duck-adapted strain of fowl plague as a predictable cause of the duck virus enteritis. He showed that chickens, pigeons, and rabbits were refractory to experimental infection. DeZeeuw suspected that wild waterfowl were carriers of the disease, as they were found within outbreak areas.

Bos (4) re-examined findings of the earlier workers and observed new outbreaks. He further characterized lesions, clinical

signs, and immune response of ducks by experimental study and was unable to reproduce the disease in chickens, pigeons, rabbits, guinea pigs, rats, or mice. He concluded that the disease was not caused by fowl plague virus, but was a new distinct viral disease of ducks, which he termed "duck plague." This conclusion was based on the high degree of specificity of the agent for ducks, both in experimental and naturally occurring infections, persistence of the disease as a uniform entity in the Netherlands, and the longer incubation period. He differentiated it from Newcastle disease. These observations were further supported by more detailed studies on virus propagation, incidence and distribution, pathology, and immunity (34, 35, 36, 37, 38, 39, 40, 41).

Since the first reports of DVE in domestic and free-flying anseriforms (ducks, geese, and swans) (49, 55), serious outbreaks in migratory waterfowl with high mortality have been reported (22). Outbreaks in zoos and game farm flocks have also been reported (33, 53, 62).

Prior to the 1973 massive outbreak in migratory waterfowl in Lake Andes, South Dakota, the United States Department of Agriculture considered the disease exotic. Because of the high prevalence of DVE in North America the disease is considered enzootic and is reportable. New outbreaks should be reported to the state veterinarian and the federal area veterinarian in charge of the affected state. For a review of the disease in North American waterfowl, see Brand (5).



13.2. Thin section of Epon-embedded cells infected 48 hours with Long Island isolate of duck enteritis virus. Virus particles (arrows) appear in several forms in the nucleus (N), cytoplasm (C), and a cytoplasmic vacuole (V). Bar = 1 μm. (7).

Etiology

Classification

The causative agent of DVE is a herpesvirus, belonging to the Alpha-herpesvirinae subfamily. Duck enteritis virus (DEV) is nonhemagglutinating (34) and nonhemadsorbing (17).

Morphology

Electron microscopy of virus-infected cells revealed virus particles in both nucleus and cytoplasm (Fig. 13.2) (7). Bergmann and Kinder (3) and Tantaswasdi *et al.* (86) studied structure and maturation of DEV in the cells of infected ducklings. They recorded spherical nucleocapsids about 91–93 nm in diameter with nucleoids (cores) approximately 61 nm in diameter in the nuclei of the host cells. Virus particles about 126–129 nm in diameter, probably the result of the envelopment of nucleocapsids by the nuclear membrane, were seen in the cytoplasm and perinuclear spaces. Larger mature particles varying in size from 156–384 nm in diameter were observed in the tubular system of the endoplasmic reticulum in the cytoplasm. These consisted of enveloped nucleocapsids encased in an osmiophilic matrix and surrounded by an additional membrane. These morphological structures differentiate DEV from other animal herpesviruses (3). In another study, four forms of nucleocapsids were identified in the tissues of infected ducks, which were found in the cytoplasm and nucleus, and measured 42–90 nm in diameter (100). Two types of nucleocapsids were associated with the intranuclear inclusion bodies; round or rod-shaped nucleocapsids containing an electron-dense internal core and nucleocapsids containing electron-dense particles in close proximity to the inner capsid wall, with an electron-lucent cross or pentagonal shape inside. Many nucleocapsids, mature viruses and viral inclusion bodies were observed in the nucleus and cytoplasm of infected cells of liver, small intestine, spleen, thymus and bursa of Fabricius.

Chemical Composition

The virion contains DNA (7). RNase treatment on thin sections had no effect on ultrastructural morphology of the virus, and exposure to DNase led to the removal of the central core without affecting the envelope. Fluorescence of intranuclear inclusion bodies in cell cultures stained with acridine orange was also consistent with the presence of DNA (30). Inactivation by pancreatic lipase indicates that the virions contain an essential lipid (30).

Virus Replication

Development of the virus in cell cultures was studied by electron microscopy and growth curves of intracellular and extracellular virus (3, 7, 86). Examination of thin sections revealed development forms only in the nucleus 12 hours postinoculation. By 24 hours, in addition to viral forms in the nucleus, larger particles with an envelope were observed in the cytoplasm. Virus titrations

of similar cell cultures demonstrated new cell-associated virus 4 hours postinoculation, with maximum titer at 48 hours. Extracellular virus was first detected 6–8 hours postinoculation and reached maximum titer at 60 hours (7). Increased incubation temperatures of tissue cultures (39.5–41.5°C) favored viral replication, especially of less virulent strains (8).

In a susceptible host, virus replicates primarily in the mucosa of the digestive tract, especially in the esophagus, and then spreads to the bursa of Fabricius, thymus, spleen, and liver. The epithelial cells and macrophages of these organs are the principal sites of viral replication (31).

Susceptibility to Chemical and Physical Agents

The virus was found to be sensitive to ether and chloroform (30). Exposing virus for 18 hours at 37°C to trypsin, chymotrypsin, and pancreatic lipase markedly reduced or inactivated it, and papain, lysozyme, cellulase, DNase, and RNase had no effect. Cells treated with DNase showed intranuclear inclusions, but there was marked reduction in fluorescence when stained with acridine orange (30).

Thermal inactivation studies (30) revealed that infectivity was destroyed after heating for 10 minutes at 56°C or 90–120 minutes at 50°C. At room temperature (22°C), infectivity was lost after 30 days. Drying over calcium chloride at 22°C resulted in inactivation after 9 days.

Exposure of the virus for 6 hours at pH levels of 7, 8, and 9 resulted in no loss of titer, but a measurable titer reduction was noted at pH 5, 6, and 10. At pH 3 and 11, the virus was rapidly inactivated. A marked difference in inactivation rates was noted between pH 10 and 10.5 (30).

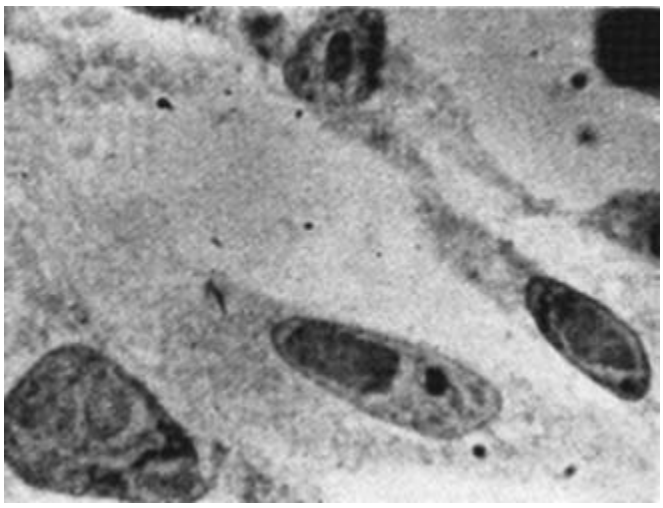
Strain Classification

Although differences in virulence among DEV strains have been noted, all appear to be immunologically identical (30, 38, 84) and antigenically related (80). The virus is immunologically distinct from other avian viruses, including fowl plague, Newcastle disease, duck hepatitis (4, 17, 39, 56), and other herpesviruses (74).

A herpesvirus was isolated from domestic geese in Australia showing postmortem appearance and histopathological changes similar to those seen in DVE (42). The virus isolate was antigenically and genomically distinct from DEV as shown by protection, serological analysis and genetic characterization by restriction endonuclease digestion and polymerase chain reaction (PCR) assays (26).

Laboratory Host Systems

Duck enteritis virus can be propagated in Pekin duck embryo fibroblasts (98) incubated at 39.5–41.5°C (8) in duck embryo liver or kidney primary cells (25), Muscovy duck embryo fibroblasts (44) and on the chorioallantoic membrane (CAM) of 9–14-day-old embryonating duck eggs (34). The virus can be adapted to grow in embryonating chicken eggs (34) and chicken embryo cell cultures (17); however, they are unsatisfactory for primary isolation. The virus produces cytopathogenic effect in inoculated cell cultures (17,46), and intranuclear inclusions have been observed in infected chicken and duck embryo cell cultures (29) (Fig. 13.3). Plaque assays have been used to measure virus and neutralizing antibody titers (17). In the presence of complement, antibodies to DEV are capable of lysing infected duck embryo fibroblasts (47).



13.3. Inclusion bodies in duck embryo fibroblasts infected with duck virus enteritis. ×10,000 (Plum Island Animal Disease Laboratory).

Pathobiology and Epidemiology

Incidence and Distribution

In addition to the Netherlands, DVE has been reported in China (40) and confirmed in France (23, 59), Belgium (19), India (64, 65), Thailand (68), England (1, 27), Canada (29, 96), Hungary (92), Denmark (69), Austria (66), and Vietnam (94).

In 1967, the first reported outbreak in North America was observed in white Pekin ducks in the concentrated duck-producing area of Long Island (54). In addition, outbreaks in wild, free-flying waterfowl occurred at seven different locations on Long Island, New York (49, 55). The disease has been reported in 21 states, with repeated outbreaks in New York, Pennsylvania (32, 81), Maryland (63), California (83), Virginia, Wisconsin and Texas. An extensive survey for duck enteritis virus (DEV) in North American wild waterfowl failed to detect the virus, indicating that the disease is not enzootic in them (6).

In the Netherlands, a higher incidence of DVE was noted in the spring (35); however, on Long Island, no seasonal increase was noted. In contrast, a higher incidence of DVE in wild, free-flying anseriforms on Long Island was observed in the fall of 1967 (49).

Natural and Experimental Hosts

Natural susceptibility to DVE has been limited to members of the family Anatidae (ducks, geese, and swans) of the order Anseriformes, although the virus can be adapted by serial passage to grow in embryonating chicken eggs and chickens up to 2 weeks of age (37, 38). Natural infections have occured in ducks ranging from 7 days of age to mature breeder. Naturally occurring out-

breaks have occurred in a variety of domestic ducks (*Anas platyrhynchos*), including white Pekin, khaki campbell, Indian runner, hybrids, and native ducks of mixed breeding. Outbreaks are quite common in Muscovy ducks (*Cairina moschata*) (34, 55). In an outbreak reported in Illinois (14), all 625 Muscovy ducks died in a flock of 650 waterfowl consisting of black ducks, rhumen ducks, Pekins, Muscovies and geese. Naturally occurring infections have also been reported in domestic geese (*Anser anser*) (41) and mute swans (43). Gray call ducks have been found to be resistant to lethal infection (91). Outbreaks of DVE in domestic ducks are frequently associated with aquatic environments cohabited by wild waterfowl (20, 55).

Susceptibility of various species of anseriforms to experimental DVE has been studied (91). In addition to domesticated species, mallards (*A. platyrhynchos*), Garganey teal (*A. querquedula*), gadwall (*A. strepera*), European widgeon (*A. penelope*), wood ducks (*Aix sponsa*), shovelers (*Spatula clypeata*), common pochards (*Aythya ferina*), common eiders (*Somateria mollissima*), white-fronted geese (*Anser albifrons*), bean geese (*A. fabalis*), and mute swans (*Cygnus olor*) were susceptible to lethal infection. European teal (*A. crecca*) and pintails (*A. acuta*) were resistant but produced antibodies against DVE as a result of experimental exposure. Mallards were more resistant to lethal effects and were considered a possible natural reservoir of infection. An experimental study (96) showed blue-winged teal (*A. discors*) and Canada geese were extremely susceptible to DEV and experienced high mortalities. Blue-winged teal had few gross lesions at necropsy.

A recent outbreak has been reported in common coots (*Fulica atra*) and crested coots (*Fulica cristata*) belonging to the family Rallidae order Gruiformes (75), but the virus was not isolated or identified. Of the order Charadriiformes, herring gulls (*Larus argentatus*) and black-headed gulls (*L. ridibundus*) were not susceptible to experimental infection and failed to produce antibodies against DEV (91).

The first reported outbreaks of spontaneous DVE in wild waterfowl were diagnosed on Long Island, New York (49, 54). It was detected in mallards, black ducks (*A. rubripes*), a Canada goose (*Branta canadensis*), a bufflehead (*Bucephala albeola*), a greater scaup (*Aythya marila*), and a mute swan.

A major epornitic of DVE occurred at Lake Andes, South Dakota, in 1973, with an estimated loss of 43,000 ducks and geese out of a total population of 100,000 (22). Duck virus enteritis was diagnosed in black ducks, mallards, pintail-mallard hybrids, redheads (*Aythya americana*), common mergansers, common goldeneyes (*Bucephala clangula*), canvasbacks (*Aythya valisineria*), American widgeon (*Mareca americana*), wood ducks, and Canada geese. A study on susceptibility of waterfowl to Lake Andes strain showed that blue-winged teals, wood ducks, and redheads were highly susceptible; muscovies and gadwalls were moderately susceptible; mallards and Canada geese were less susceptible; and pintails were the least susceptible (85).

Transmission, Carriers, and Vectors

Duck virus enteritis can be transmitted by direct contact between infected and susceptible birds or indirectly by contact with a contaminated environment. Because waterfowl are dependent on an aquatic medium to provide a common vehicle for feeding, drinking, and body support, water appears to be the natural means of virus transmission from infected to susceptible individuals. Support for this concept is found in the history of new outbreaks in domestic ducks, which have been limited to birds having access to open bodies of water cohabited by free-flying waterfowl. After infection is established, it can be maintained in the absence of open water or infected birds if susceptible populations are moved onto recently contaminated premises.

New foci of infection may be established by movement of infected waterfowl into susceptible flocks or onto bodies of water previously free of virus contamination. Course and direction of the infection are defined by population densities and rate of transmission between infected and susceptible waterfowl. Population densities in concentrated duck-producing areas encourage rapid spread of DVE, with high mortality. Breeder ducks usually are selected and placed in a defined area and maintained in the same location for the balance of their productive lives. Once a breeder population is exposed, DEV is self-limiting. In contrast, market ducks are progressively moved as they mature and are relocated in areas formerly occupied by the next oldest age group. Infection in market ducklings tends to be a continuous recycling as susceptible birds are sequentially moved to contaminated environments.

Experimentally, DEV can be transmitted via oral, intranasal, intravenous, intraperitoneal, intramuscular, and cloacal routes. Although the intramuscular route of inoculation requires the least amount of virus to kill the inoculated birds, the intranasal and conjunctival routes require more virus, and the oral route requires the most virus (85). Potential transmission by bloodsucking arthropods may be possible during viremia. Although virus has been isolated from an egg removed from the cloaca of an infected domestic duck (37), it has not been recovered from eggs laid during a naturally occurring outbreak. Experimental vertical transmission has been reported in persistently infected waterfowl (9).

A carrier state has been suspected in wild ducks (12, 20, 82, 91). Recovered birds become carriers and shed the virus periodically (11). Like other herpesviruses, DEV latency and reactivation have been blamed for precipitating outbreaks in domestic and migrating waterfowl populations. A recent study has revealed that trigeminal ganglion is the latency site for the virus (79). The virus was reactivated *in vitro* from trigeminal ganglia and lymphocytes. Immunosuppression with corticosteroid was shown to reactivate the virus *in vivo*.

Incubation Period

In domestic ducks, the incubation period ranges from 3–7 days. After overt signs appear, death usually follows within 1–5 days.

Clinical Signs

In domestic breeder ducks, sudden, high, persistent flock mortality is often the first observation. Mature ducks die in good flesh. Prolapse of the penis may be evident in dead mature males. In laying flocks, a marked drop in egg production may be noted during the period of highest mortality.

As infection progresses within a flock, more signs are ob-

served. Photophobia, associated with half-closed pasted eyelids, inappetence, extreme thirst, droopiness, ataxia, ruffled feathers, nasal discharge, soiled vents, and watery diarrhea appear. Affected ducks are unable to stand; they maintain a posture with drooping outstretched wings and head down suggesting weakness and depression. Sick ducks forced to move may show tremors of head, neck and body.

Young ducklings 2–7 weeks of age show dehydration, loss of weight, blue beaks, conjuctivitis, lacrimation, nasal exudate, and often a blood-stained vent.

Morbidity and Mortality

Total mortality in domestic ducks may range from 5–100%. Since the birds showing clinical signs usually die, morbidity closely approaches mortality. Adult breeder ducks tend to experience higher mortality than young ducks. No differences in mortality rates were found in mallard and white Pekin ducks experimently infected with DEV and *Riemerella anatipestifer,* indicating that these organisms do not act synergistically (61). However, mallards immunosuppressed with cyclophosphamide and challenged with a sublethal dose of DEV had higher mortality (24). Secondary bacterial infections with *Pasteurella multocida, Riemerella anatipestifer*, and *E. coli* were often seen in a natural outbreak of a low virulent strain in young ducklings as a result of immunosuppressive effect of the virus (81).

Pathology

The specific pathologic response to DEV is dependent on species affected (49); age, sex, and susceptibility of the affected host; stage of infection; and virulence and intensity of virus exposure (54, 55).

Gross Lesions

Lesions of DVE are associated with disseminated intravascular coagulopathy and necrotic degenerative changes in mucosa and submucosa of gastrointestinal tract in lymphoid and parenchymatous organs. These collective lesions, when present, are diagnostic of DVE.

Petechial, ecchymotic, or larger extravasations of blood may be found on or in the myocardium and other visceral organs and their supporting structures, including the mesentery and serous membranes. On the epicardium, especially within coronary grooves, closely packed petechiae give the surface a red "paintbrush" appearance (Fig. 13.4A). The latter lesion is observed more frequently in mature breeder ducks than in young ducklings. When heart chambers are exposed, endocardial mural and valvular hemorrhages may also be observed.

Surfaces of liver, pancreas, intestine, lungs, and kidney may be covered with petechiae. In mature laying females, hemorrhages may be observed in deformed, discolored ovarian follicles, and massive hemorrhage from the ovary may fill the abdominal cavity. Lumina of intestines and gizzard are often filled with blood. The esophageal-proventricular sphincter appears as a hemorrhagic ring.

Specific digestive mucosal lesions are found in the oral cavity (12), esophagus, ceca, rectum, and cloaca. Each of these lesions undergoes progressive alterations during the course of the disease. Initially, macular surface hemorrhages appear, which are later covered by elevated, yellow-white crusty plaques. Subsequently, the lesion becomes organized into a green superficial scab devoid of its former hemorrhagic base. Lesions range in size from approximately 1–10 mm in length. In the esophagus and cloaca, lesions may become confluent; however, close inspection will often reveal their composite structure. In the esophagus, macules occur parallel to longitudinal folds. When macular concentrations are numerous, small lesions may merge to form larger ones covered with a patchy diphtheritic membrane (Fig. 13.4B). In young ducklings, individual lesions in the esophagus are less frequent; sloughing of the entire mucosa is more common, and the lumen becomes lined with a thick yellow-white membrane. Oral erosions can be found at openings of sublingual salivary gland ducts in chronically infected waterfowl (12). Meckel's diverticulum may be hemorrhagic and contain a fibrinous core (72).

In ceca, macular lesions are singular, separated, and well defined between mucosal folds. The external surface of affected ceca often presents a barred, congested appearance.

Rectal lesions are usually few in number with greatest concentration at the posterior portion of the rectum, adjacent to the cloaca.

In the cloaca, macular lesions are densely packed; initially, the entire mucosa appears reddened. Later, individual plaque-like elevations become green and form a continuous scale-like band lining the lumen of the organ.

All lymphoid organs are affected. The spleen tends to be normal or smaller in size, dark, and mottled (Fig. 13.4E). The thymus is atrophied and has multiple petechiae (Fig. 13.4F) and necrotic focal areas on the surface and cut section and is surrounded by clear yellow fluid that infiltrates and discolors subcutaneous tissues of the adjacent cervical region from the thoracic inlet to the upper third of the neck. The latter lesion is of importance in meat inspection and is easily detected when the opened neck of the carcass is observed on the processing line. The bursa of Fabricius is intensely reddened during early infection (Fig. 13.4C). The exterior becomes surrounded by clear yellow fluid that discolors adjacent tissue of the pelvic cavity. When the lumen of the bursa is opened, pinpoint yellow areas are found in an intensely hemorrhagic surface. Later, walls of the bursa become thin and dark, and the bursal lumen is filled with white coagulated exudates (Fig. 13.4C). Intestinal annular bands appear as intensely reddened rings visible from external and internal surfaces. Yellow pinpoint areas can be observed on the mucosal surface. Later, the entire band becomes dark brown and tends to separate at its margins from the mucosal surface. The multifocal necrosis of gut-associated lymphoid tissue causes ulceration covered by fibrinous pseudomembranes (Fig. 13.4D).

During early stages of infection, the entire liver surface has a pale copper color with an admixture of irregularly distributed pinpoint hemorrhages and white foci (Fig. 13.4E), giving it a heterogeneous, speckled appearance. Late stages of infection are characterized by dark bronze or bile-stained livers without hemorrhages; the white foci are larger and appear more distinct on the darker background.

Although these lesions are consistent with DEV infection, each age group responds distinctively. In ducklings, tissue hemorrhages are less pronounced and lymphoid lesions are more prominent. In mature domestic ducks with naturally regressed bursa of Fabricius and thymus, tissue hemorrhages and reproductive tract lesions predominate.

In geese, intestinal lymphoid disks (51) are analogous to annular bands in ducks. In a single Canada goose, lesions of the intestinal lymphoid disks resembled "button-like ulcers" (50). Similar intestinal lesions have been observed in an outbreak of DVE in Canada and Egyptian geese. In swans, diphtheritic esophagitis is a consistent lesion (43).

An outbreak caused by a low virulent strain of DEV in commercial 2–6-week old white Pekin ducklings produced atypical gross lesions, including diphtheritic membranes under the tongue and in nasal and infraorbital sinuses. Esophageal mucosa had a few necrotic plaques, and cloacal mucosa was covered with necrotic greenish diphtheritic membranes. No lesions were seen in the intestines including annular bands. Thymus and bursa were atrophied and hemorrhagic (Fig. 13.4F), and the bursal lumen was filled with cheesy exudate. An experimental study showed that the bursal atrophy could last for at least 39 days postinfection; however, the thymus recovered after 10 days of infection (81).

Microscopic Lesions

The initial lesion occurs in the walls of blood vessels. Smaller blood vessels, venules, and capillaries, instead of larger blood vessels, are more markedly involved. The endothelial lining is disrupted, and connective tissue of the wall becomes less compact, with visible separations at points where extravasations of blood pass from the lumen through the thin ruptured wall into surrounding tissues.

Hemorrhages are especially pronounced in certain locations: interlobular venules of the proventriculus, hepatic and portal venules at the margins of liver lobules, venules in the spaces between lung parabronchi, capillaries within intestinal villi, and star-shaped intralobular renal hemorrhages.

As a result of vascular damage, affected tissues undergo progressive degenerative changes. Microscopic changes can be found in any visceral organs including those without gross lesions.

Digestive lesions appear initially as hemorrhages of capillary arcades of submucosal papillae or folds. Hemorrhages become larger and confluent, elevating and separating the overlying mucosa. The affected epithelium above the hemorrhage becomes edematous, necrotic, and raised into the lumen above normal adjacent mucosal surfaces (Fig. 13.4G). Later, margins of necrotic epithelium separate to define the borders of elevated plaques. There is necrosis and degeneration of stratified squamous epethelium of the esophagus and cloaca (71). Eosinophilic intranuclear and cytoplasmic inclusions have been seen in epithelial cells (81, 86).

Hemorrhage from venules and capillaries fills lymphoid tissue within intestinal annular bands or lymphoid disks and lymphoid tissue of the esophageal-proventricular sphincter and spleen. Lymphocytes undergo karyorrhexis and pyknosis. Fragments of lymphocytes appear everywhere and are engulfed by phagocytes. In addition to cellular debris and hemorrhage within lymphoid follicles, marked swelling of reticulum cells occurs, and their cytoplasm becomes subdivided and condensed into spherical and oval pale-staining bodies. Reticulum cells rupture and discharge their cytoplasmic contents into tissue spaces. An intranuclear inclusion body and delicate nuclear membrane and cell wall are the remaining vestiges of reticulum cells.

Intestinal lymphoid lesions become large hemorrhagic infarcts. A layer of free blood separates lymphoid tissue from the mucosa, which undergoes coagulation necrosis. The necrotic mucosa forms a pseudomembrane higher than adjacent normal intestinal mucosa.

In the small intestines, sheets of epithelial cells are displaced from the surface of villi, many of which are broken and cast into the lumen. Abundant blood and cellular debris fill the lumen.

Within the bursa of Fabricius, submucosal and interfollicular capillary hemorrhages are found. There is a severe depletion of lymphocytes in the follicles, many of which have empty hollow cavities in the medulla. Corticomedullary epithelial cells, capillary networks, and large phagocytic cells containing fragmented lymphocytes form the circumference around these cavities. Severe depletion of lymphocytes in the follicles occurs, which is replaced by eosinophilic material mixed with heterophils. There are occasional mononuclear cells that contain intranuclear inclusions. Bursal epithelial cells are hypertrophied with vacuolated cytoplasm and contain both intranuclear and intracytoplasmic inclusions (81).

In the thymus, free blood fills interfollicular spaces. Coagulation necrosis of central medullary reticulum cells and destruction of cortical lymphocytes are pronounced.

In mature female breeder ducks, congestive, hemorrhagic, and necrotic alterations occur in the oviduct. Follicles may be misshapen and blood stained. In the ovary of immature female breeder ducks, focal intestinal hemorrhages from capillaries and venules may be found.

In mature breeder drakes, focal capillary hemorrhages occur in interstitial tissues between seminiferous tubules. In parenchymatous organs such as liver, pancreas, and kidneys, hemorrhages and focal necrosis are found surrounding blood vessels.

Within necrotic foci in the liver, hepatic cords show a variety of changes including detachment and disassociation of hepatocytes from each other and their surrounding structure. A few necrotic liver cells become swollen or subdivided and discharge their cytoplasmic contents through a ruptured cell surface and are represented only by intranuclear inclusion bodies. Focal areas of necrosis may be filled with fibrin (Fig. 13.4H). Similar, but more limited, changes occur in pancreas and kidney (52).

Immunity

Active

Active immunity has been demonstrated following the use of a modified live-virus vaccine (37) and inactivated tissue culture vaccine (80). It is assumed that both humoral and cell-mediated immunity are involved in protection (48, 89). Field observations suggest that recovered birds are immune to re-infection with DEV.

Passive

Maternal immunity has been reported in ducklings, but it declines rapidly. Progeny of vaccinated breeder ducks with a live-attenuated virus vaccine are fully susceptible. However, ducklings from breeders that had been vaccinated and challenged with a virulent virus were fully protected at 4 days of age, and less than 40% were protected at 13 days of age (88). In an experimental study (10), superinfection of persistently infected mallard ducks resulted in death, indicating that protection against mortality was dependent on the route of exposure, strain of the initial virus, and strain of superinfecting virus.

Diagnosis

Isolation and Identification of DEV

Although a presumptive diagnosis can be made on the basis of gross and histopathologic lesions, isolation and identification of DEV confirms the diagnosis even in the absence of typical lesions. Samples recommended for virus isolation are liver, spleen, bursa, kidneys, peripheral blood lymphocytes (PBL) and cloacal swabs. Virus isolation is carried out by inoculation of susceptible 1-day-old Muscovy, white Pekin ducklings, or onto CAM of 9-14-day-old embryonating duck eggs. Characteristic lesions and mortality in inoculated ducklings are highly suggestive of DVE. Virus can also be isolated and propagated in white Pekin or Muscovy duck embryo fibroblasts, and primary cell culture of liver and kidney. Virus identification is carried out by virus neutralization assay in embryonating duck eggs or cell culture using DEV-specific antiserum.

Conventional polymerase chain reaction (PCR) assay is used for detection of DEV DNA in tissue samples and in cell culture (28, 67, 70). Recently, a quantitative real-time PCR assay has been developed that may be useful for rapid diagnosis and detection of DEV DNA in acute and latent stages of infection (99). Latex agglutination test was claimed to be as sensitive as duck embryo inoculation and virus neutralization in the detection of viral antigens (15). Restriction endonuclease analysis could be a possible test for differentiation of DEV strains (93).

Serology

Increase in virus neutralization (VN) titers following convalescence from DVE will demonstrate progress of the disease within a flock. A VN index of 1.75 or more indicates infection with DEV (16). A VN index of 0–1.5 has been found in sera of domestic and wild waterfowl not exposed to the disease. The use of chicken embryo-adapted virus in chicken eggs for VN studies is safer and more convenient than the use of field-strain viruses inoculated onto the CAM of duck eggs (16). Immunofluorescence tests can be used to detect viral antigens in cell cultures or tissue sections (21, 78). Other serologic procedures for detecting antibodies include a microtiter plate isolation and neutralization test using duck embryo fibroblasts (97), a reverse passive hemagglutination test (18), and enzyme-linked immunosorbent assay (ELISA) (45, 77). A Dot-ELISA and passive hemagglutination assays have been developed for detection of DEV antibodies, however, the specificity and sensitivity of these two assays were shown to be moderate (60).

Differential Diagnosis

Differential diagnosis requires consideration of other diseases producing hemorrhagic and necrotic lesions in anseriforms. In domestic ducks, common diseases producing such changes are duck virus hepatitis, fowl cholera, necrotic enteritis, coccidiosis, and specific intoxications. Although Newcastle disease, fowl pox, and fowl plague are reported to produce similar changes in anseriforms, these diseases have been infrequently reported.

Intervention Strategies

Management Procedures

Prevention is achieved by maintaining susceptible birds in environments free from exposure to the virus. These measures include the addition of stock known to be free from infection and avoiding direct and indirect contact with possibly contaminated material. Introduction of the disease by free-flying anseriforms and contaminated aquatic environments must be prevented. All possible measures should be taken to prevent dissemination of virus by free-flowing water. After DVE has been introduced, control can be effected by depopulation, removal of birds from the contaminated environments, sanitation, disinfection, and vaccination of all susceptible ducklings.

In countries where the disease is not enzootic and is truly exotic, measures should be taken to further prevent entry and dissemination into geographic areas known to be free from DVE. This would include specific examination to prevent importing infected anseriforms. Accordingly, there would be surveillance of ornamental bird collections, zoos, and domestic growers of anseriforms. Efforts should be made to provide efficient detection of DEV by laboratory workers and waterfowl specialists so that its presence, status, and importance can be better defined.

Vaccination

Vaccination has been used as a preventive measure and also for controlling disease outbreaks.

Inactivated vaccines have been tried but have not been as efficacious as modified live-virus vaccines (13). However, an inactivated tissue culture-grown virus vaccine has been shown to provide protection against the virulent strain (80). This vaccine could be used in domestic and captive waterfowl without the risk of introducing a live virus.

A chicken embryo-adapted DEV strain, avirulent for domestic ducks, has been developed and used extensively with good success in the Netherlands (37). This vaccine strain has also been used to prevent and control DVE outbreaks on commercial duck farms and captive waterfowl collections in the United States and Canada (62, 76). The vaccine can be used in the face of an outbreak, as it provides protection immediately after vaccination due to an interference phenomenon (36, 73). It should be noted, however, that birds in the period of incubation may not be protected. A naturally apathogenic and immunogenic strain of DEV was reported to be successful for active and passive immunization of ducks (57, 58).

Attenuated live virus vaccine is administered by subcutaneous or intramuscular routes in domestic ducklings more than 2 weeks

of age. Normally, the breeding flocks are vaccinated. Flocks maintained for more than a year are revaccinated annually. Apparently, vaccinated ducklings do not excrete inoculated virus to a degree that would be sufficient to bring about contact immunization (38, 87).

Treatment

There is no specific treatment for infection with DEV.

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Hemorrhagic Nephritis Enteritis of Geese (HNEG)

J-L. Guérin

Introduction

Hemorrhagic nephritis enteritis of geese (HNEG) is one of the major diseases of geese in Europe. HNEG had been named for a long time "young geese disease", or "late form of Derzsy's disease," by confusion with goose parvovirus infection. According to its etiology, a more relevant denomination should be "goose polyomavirosis." This systemic, frequently lethal disease is also the only polyomavirus infection described to date in a poultry species.

Public Health Significance

Polyomaviruses are supposed to have a very narrow host range (20). This is supported by recent evidence of co-divergence of mammalian and avian polyomavirus with their respective hosts (13). HNEG is therefore supposed to have no public health implication.

History

HNEG was first described in 1969 in Hungary (2), where occurrence of field cases was always associated with administration of serum—collected from convalescent flocks formerly affected by Derzsy's disease—to young geese, in order to confer to them passive immunity. No spontaneous case of HNEG has been reported so far in this country. HNEG was described a few years later in Germany (17), then in France (18, 22), where it evolved with a sporadic pattern, except epizootics in the late 80's and since 1997 (5). For many years, HNEG was suspected to correspond to a late evolution of Derzsy's disease. Actually, since hyperimmune sera against goose parvovirus or duck hepatitis virus did not protect goslings from HNEG, it appeared clearly that HNEG agent was a distinct virus (10). Etiology of HNEG was clarified lately by Guérin *et al.* (6), exactly 30 years after the first clinical report.

Etiology

Classification

The agent of HNEG, namely goose hemorrhagic polyomavirus (GHPV), is member of the Polyomaviridae family and Polyomavirus genus (6). The budgerigar fledgling polyomavirus (BFPyV) is the prototype avian polyomavirus, infecting psittacines, falconiforms and passerines (3, 4, 8, 15).

Morphology

Virus particles are naked, spherical and show icosahedral symetry. Their size ranges from 40 to 50 nm in diameter (6). Buoyant density of virions is of 1.20 $g.cm^{-3}$ in sucrose gradient (6), which corresponds to 1.34–1.35 $g.cm^{-3}$ in CsCl.

Chemical Composition

GHPV genome is a circular, double stranded DNA of 5256 pb. Genome organization of all polyomaviruses shares common features, with a set of early genes encoding polymerases (t and T antigens) and late genes, encoding structural proteins: VP1, the main capsid protein, and two other structural proteins, VP2 and VP3 (9). As for avian polyomaviruses, an additional VP4 has been evidenced, the precise functions of which still remain to be clarified (9).

Virus Replication

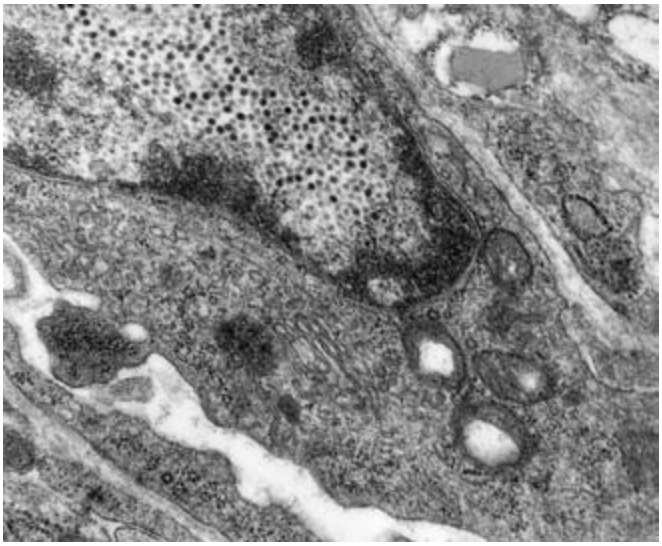
Replication of GHPV occurs in the nucleus: observations of infected cells show a huge concentration of viral material in the nucleus, either in culture cells or tissues of infected goslings (Fig.13.5) (6). Virus is easily detected in the nuclei by immunofluorescence, using serum of geese recovering from HNEG (Fig. 13.6). Releasing of virions implicates disruption of cell membrane.

Susceptibility to Chemical and Physical Agents

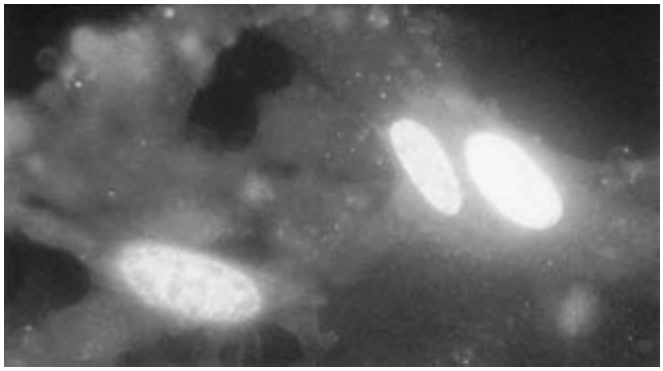
GHPV shows a great resistance to heating: virus is still fully virulent after a 2 hours incubation at 55°C (6). The virus also is resistant to freezing-thawing cycles and to lipids solvents: treatment with 1% phenol has no effect on its viability (10). The other avian polyomavirus, BFPyV, is mostly sensitive to chloride derived products (16).

Strain Classification

Genetic variability among field isolates has not been assessed so far. Nevertheless, polyomavirus genomes are supposed to be highly conserved: all the variants of BFPyV isolated from psittacines, falconiforms or finches share more than 99% nu-



13.5. Electron micrograph of a GHPV-infected cell. Notice many naked virions in the nucleus and peripheral accumulation of chromatin. ×25,000.



13.6. Immunostaining of goose polyomavirus in kidney cell culture. Intranuclear replication is evidenced by indirect immunofluorescence, using isothiocyanate fluoresceine-labelled antibody.

cleotide identity (14). Phylogenic analysis of GHPV confirmed that VP1 is remarkably conserved among isolates from different countries (12). No cross-neutralization experiment has been performed so far on GHPV field isolates.

Laboratory Host System

HNEG is successfully reproduced by parenteral inoculation on 1-day-old goslings. Death occurs between 6 to 8 days post-inoculation, with a peracute evolution. Goslings are susceptible to inoculation by either subcutaneous or intraperitoneal routes. All attempts to adapt the HNEG virus to duck fibroblasts or embryos remain unsuccessful (6, 7). Goose fibroblasts are also refractory to virus replication (6). Propagation of GHPV on goose embryos has been reported: 14-day-old goose embryos inoculated onto the chorioallantoid membrane (CAM) died from 8 to 10 days post-infection, with lesions similar to those described in goslings (1). Virus propagation can be accomplished by propagation on epithelial primary cells derived from 1-day-old gosling kidneys. A cytopathic effect appears by day 5 post-inoculation: granulations and vesicles are distinguished in the cytoplasm, followed by budding of the cell, and finally cell detachment from the monolayer (6). Cell-based dilution titration procedures are seldom done, since cytopathic effect appears late after infection. Alternatively, detection and quantification of virus yields from cell cultures could be assessed by quantitative real-time PCR (7).

Pathobiology and Epidemiology

Incidence and Distribution

Until now, HNEG has been described in Hungary, Germany and France (2, 5, 17, 18, 22). Occurrence in other countries seems likely, although there's no published report confirming this clearly. Cases are frequently observed in winter, probably due to climatic conditions or weakness of the goslings hatched from light-conditioned breeders (5, 7).

Natural and Experimental Hosts

HNEG has only been described to date in growing geese. Unapparent infections have been evidenced in migrating wild geese (7). Other waterfowl species, such as mule or Muscovy ducklings are clinically refractory to GHPV inoculation (7). Actually, reproduction of HNEG signs in ducklings, reported in 1970 by Szalai and Bernath (21) could never be confirmed. In the same way, no HNEG-like syndromes have ever been observed in Pekin, mule or Muscovy ducks.

Transmission, Carriers, Vectors

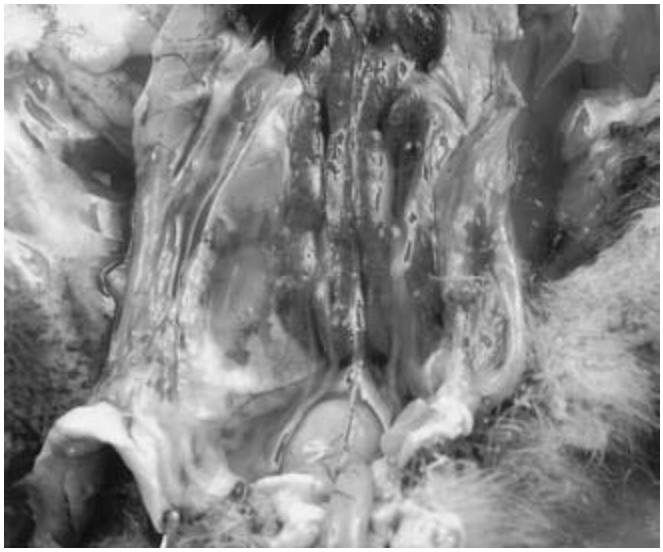
Infected birds excrete amounts of virus in their droppings, resulting in dissemination of contagious material in the environment, and easy direct and indirect contaminations. Vertical transmission of the virus through the egg has never been confirmed so far but can not be excluded. The experimental infection of goose embryos has been evidenced, but it does not formally demonstrate a field occurrence of vertical transmission (1). No biologic vector seems to be involved in GHPV transmission.

Incubation Period

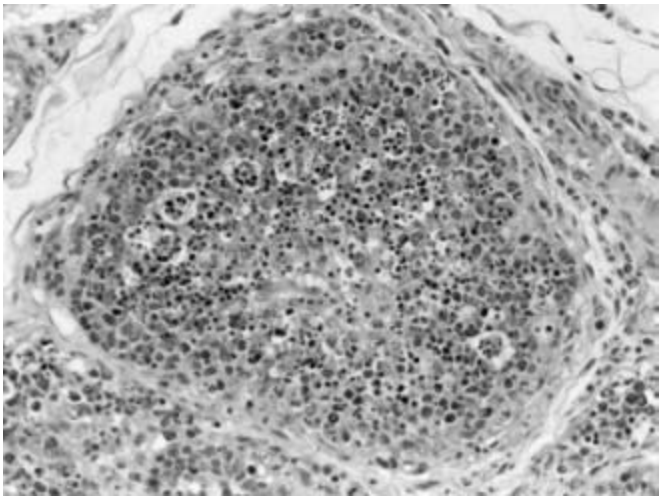
Incubation period is mostly age dependent. Inoculation of day-old goslings results in death within 6 to 8 days. In contrast, in 3-week-old goslings, incubation lasts for up to 15 days (12). After 4 weeks, inoculation results mostly in non-clinical infection. Actually, although clinical signs rarely start before 5 or 6 weeks of age, contamination likely occurs early in life, as observed for other polyomaviruses (15).

Clinical Signs, Morbidity, and Mortality

HNEG has been described in goslings from 4 to 10 weeks old. In affected flocks, morbidity ranges from 10 to 80% and death is the most common outcome (5). Clinical signs develop only few hours before death: birds sit alone, away from the flock, stay in a coma and die (2, 10). Nervous signs, such as opistothonos, are



13.7. Gross lesions in a 10-day-old gosling infected with goose polyomavirus. Note edema, gelatinous ascites, and swelling of kidneys.



13.8. Microscopic lesions in a follicle of cloacal bursa. Severe lympholysis in the center of the follicle and in cortical foci (arrow). H & E, ×150.

quite only observed after experimental or iatrogenic infections of goslings (5). Chronic evolution of the disease leads to urate deposits on viscera and in joints, resulting in lameness. In these late forms, mortality may be limited to few birds every day, up to the age of 12 weeks.

Pathology

Necropsic findings include edema of subcutaneous connective tissues, gelatinous ascites, inflammation of the kidneys (Fig. 13.7) and less frequently, hemorrhagic enteritis. Renal dysfunction leads to an increase of blood uric acid concentration (17): geese which die after a chronic evolution show visceral gout and urates in the joints (10, 11, 19). Histopathologically, the most obvious features are (i) an interstitial nephritis and necrosis of the kidney tubular epithelium (10, 11) and (ii) a moderate to severe lymphocytosis in cortical and medullar regions of follicles of the cloacal bursa (Fig. 13.8), suggestive of B-lymphocyte depletion (6, 11). Gross lesions of enteritis are associated with necrosis of intestinal epithelium. Hemorrhagic foci are also observed in most tissues, particularly in acute infections (11, 12). No inclusion could be detected in tissues of birds diagnosed with HNEG (6, 11). Electron microscopy examination of infected tissues shows aggregated virions in nuclei (Fig. 13.5) and large vesicles of dense material, including optically clear centers, in the cytoplasm of about 20% of the infected cells, either in culture cells or goose tissues (6).

Pathogenesis of the Infectious Process

During the course of infection, GHPV seems to replicate first in endothelial cells, nuclear enlargement of endothelial cells and arteriolitis being the first lesions noticed (11, 12). These histological findings suggest a selective tropism for endothelial cells, which might be of great relevance in pathogenesis of HNEG. Endothelial cells are indeed known to play a critical role in many biological pathways, resulting in vascular dysfunctions as ascites or edema. Another main target of GHPV is lymphoid cells: virions are observed in many bursal lymphoid cells and cloacal bursa systematically shows a significant lympholysis, whereas thymus lymphoid cells are less or not affected. This feature is fairly relevant with the well documented tropism of polyomaviruses to B-lymphocytes (20) and suggests an immunodepressive effect of unapparent infections.

Immunity

Immunological aspects of HNEG have so far received little attention. Neutralizing antibodies are detected in previously infected birds and their transmission to the progeny seems very efficient, since goslings hatched from infected breeders are refractory to experimental infections with huge viral load (7, 21). Duration of immunity has not been determined.

Diagnosis

Isolation and Identification of Causative Agent

GHPV can be detected in clinical material of birds putatively diseased, or in long-time, nonclinical carrier geese. Isolation could be based on either kidney cell culture (5) or goose embryos inoculation (1), but these methods are time consuming and seldom can be applied to routine diagnostic of HNEG. Detection of GHPV genome is therefore a more reliable way to detect the virus: PCR detection of DNA extracted from infected tissues (liver, spleen, kidney) with primers designed on VP1 gene is efficient and reliable (6). In non-apparent carriers, PCR assays can be advantageously performed on blood samples, spleen or cloacal swabs (7). Serology appears of poor interest to detect infection by a polyomavirus, since serologic response is greatly variable; in contrast, virus persists in infected birds for months, if not years (17).

Differential Diagnosis

Lesions of ascites, subcutaneous edema, visceral urates, nephritis in 4- to 10-week-old goslings are suggestive of HNEG. Similar lesions may however be associated with goose parvovirus. Histopathological, virological or serological procedures may be helpful in clearing etiology. Actually, HNEG is probably underdiagnosed, related to confusion with Derzsy's disease.

Intervention Strategies

Management Procedures

Goose polyomavirus spreads from carriers and clinically affected birds, mostly by fecal route (5, 10). Disinfection procedures should be thoroughly observed: complete removal of organic material, followed by the use of an appropriate disinfectant is required to prevent or interrupt a disease outbreak. Chloride derived products are considered efficient to inactivate polyomaviruses, but are particularly sensitive to the presence of organic debris (16). Since infected birds have a viremia, needles used for administration of vaccines should be sterilized between uses. Though transmission of HNEG virus through the egg is not clarified, sanitary rules should be respected in the hatchery, so as to limit early contamination of goslings, before they reach the farm. When goslings are infected by the virus, occurrence of clinical signs may greatly depend on management failures, resulting in chill and/or stress. In the same way, oil-adjuvanted vaccines should be administered with extreme caution to flocks affected by the disease.

Vaccination

Management procedures are unlikely to be sufficient for the control of HNEG infection. Vaccination of breeders could be indicated to provide maternal immunity to goslings, when they are critically sensitive to virus contamination (5). An inactived vaccine is currently under trials (7). The experimental vaccination schedule relies on (i) administration to breeders before each laying period and (ii) vaccination of growing goslings, so as to induce an active immunization covering the whole economic life of birds.

Treatment

There is no effective treatment. Prevention of stress may be helpful in preventing non-clinically infected birds from developing HNEG.

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Parvovirus Infections

Richard E. Gough

Introduction

Goose parvovirus (GPV) infection, variously known as Derzsy's disease, so-called goose influenza, goose or gosling plague, goose hepatitis, goose enteritis, infectious myocarditis, and ascitic hepatonephritis, is a highly contagious disease affecting young geese and Muscovy ducks (*Cairina moschata*). The diverse names given to the condition reflect the multiple pathological features of the disease. Depending on the age of affected goslings the disease may be present in either acute, subacute, or chronic forms (12, 67, 73). The acute form of the disease can result in 100% mortality in goslings under 10 days of age. An antigenically distinct parvovirus causing up to 80% mortality in Muscovy ducklings has also been reported from various countries. This strain of Muscovy duck parvovirus (MDPV) is much less pathogenic than goose parvovirus (28). Apart from geese and Muscovy ducks, the disease has not been reported in other avian species or mammals, including humans.

Economic Significance

In countries where geese and Muscovy ducks are farmed intensively the disease is of serious economic significance. Vaccination of breeding stock has significantly reduced the impact of the disease. However, in areas such as China where goose farming contributes to the rural economy and also provides a source of feathers and down for clothes and bedding, losses due to GPV can have a more profound social impact.

Public Health Significance

There are thought to be no public health risks associated with parvovirus infection of geese and Muscovy ducks.

History

The first detailed description of a serious disease of goslings, which occurred in China in 1956 and was later shown to be caused by a parvovirus, was reported by Fang and Wang in 1981 (22) and later confirmed by Zheng *et al.* (92). During the 1960's a similar disease was reported from many European countries, including Poland (83), W.Germany (54), Hungary (51), Bulgaria (2), Holland (10), France, USSR, and Czechoslovakia (16). Initially, many authors referred to the disease as "goose influenza," which caused some confusion as this name had originally been used for a disease of geese thought to be caused by a hemophilic bacterium (16). To distinguish the two diseases it was suggested that the "new" disease be known as "so-called goose influenza" (19). During the following years the disease was reported from all the major goose and Muscovy duck farming countries of Europe and a variety of names were given to the condition.

Although several viruses had been implicated, it was not until 1971 that Schettler (75) confirmed that the disease was caused by a parvovirus. In 1978 it was recommended that the disease be called goose parvovirus (17). Formerly, parvoviruses originating from geese and Muscovy ducks were thought to be antigenically closely related. However, following the emergence of a more virulent Muscovy duck strain of the virus, studies using virus neutralisation and molecular analysis have demonstrated significant differences between parvovirus isolates from geese and Muscovy ducks (3, 7, 56, 91).

Etiology

During the past 20 years several etiological agents have been proposed for the disease. Some early reports attributed the disease to reoviruses (14, 18, 21). It was also suggested that adenoviruses were the etiological agents as they were frequently isolated or detected from outbreaks of disease in goslings (13, 38, 68). However, in subsequent, more detailed studies, it has been confirmed that the etiological agent is a parvovirus (12, 15, 29, 49, 52, 75).

Classification

The virus was originally classified as an autonomous parvovirus belonging to the family *Parvoviridae*. More recently molecular studies have indicated that GPV is more closely related to the human *Dependovirus* genus (6, 90). No antigenic relationship with chicken or mammalian parvoviruses have been demonstrated (15, 45, 64).

Morphology

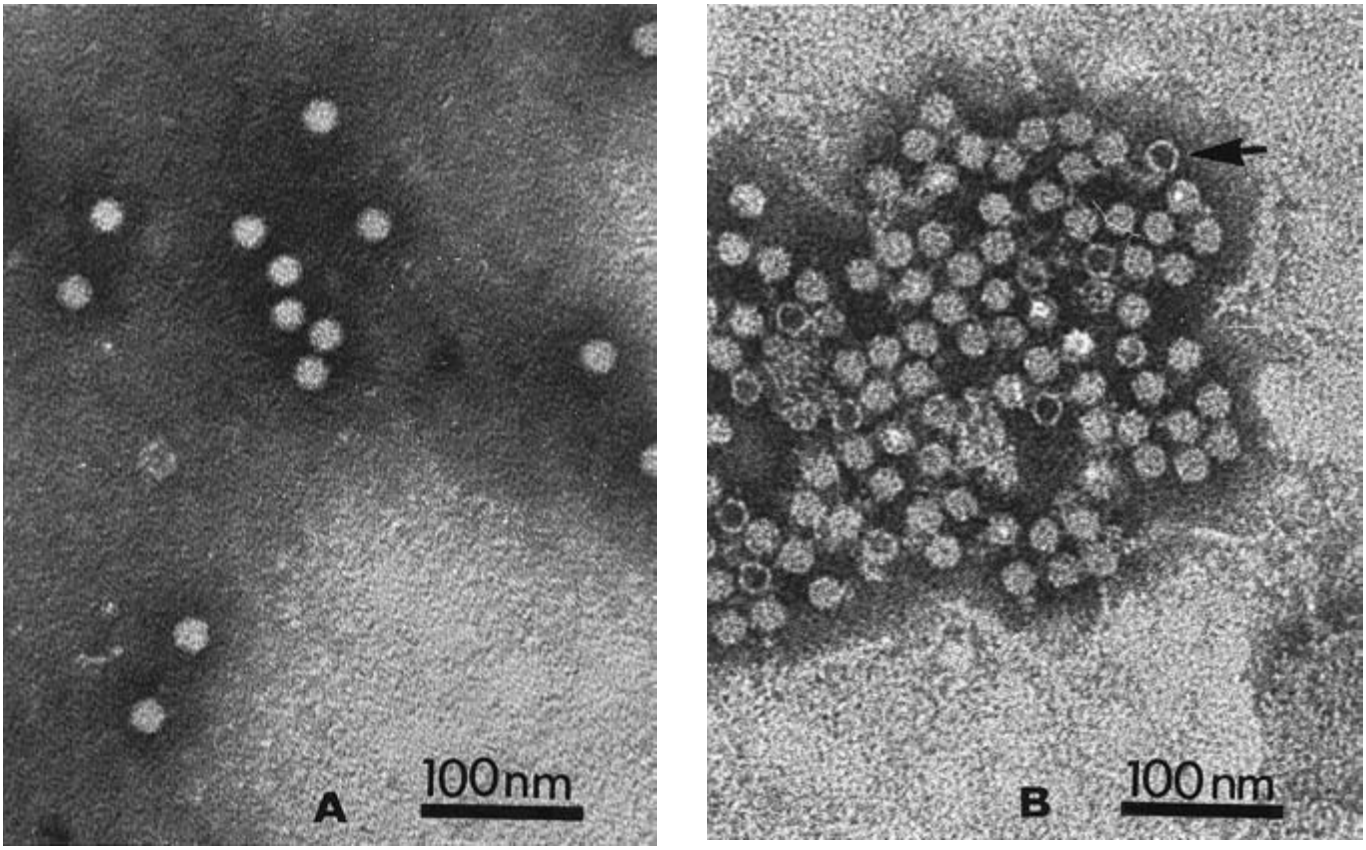
Intact virions are unenveloped and hexagonal in shape (Fig. 13.9) with an estimated 32 capsomeres and a diameter of 20–22nm (15, 29, 49, 75). The density of the virus in cesium chloride is approximately 1.38g/ml (42, 75).

Chemical Composition

Goose parvovirus, like its mammalian counterparts, has a single-stranded DNA genome (49), 5–6 kilobases in length (56, 90). Analysis of goose isolates from Bulgaria and Russia demonstrated four viral proteins with molecular weights of 88, 77, 65 and 60kD respectively (1). Similar results have been reported from Japan and Hungary using Muscovy duck parvoviruses (81, 90). Unlike several mammalian parvoviruses, haemagglutination activity, using a variety of red blood cells under different conditions, has not been demonstrated with goose parvoviruses (75).

Virus Replication

The replication of GPV has not been investigated in detail although *in vitro* studies by Kisary and Derzsy (49) have shown that viral replication takes place in the nucleus. Electron microscopy (EM) studies by Bergmann (4) have demonstrated the presence of large aggregates of parvovirus in the nuclei of cells from the heart and bursae of infected goslings. Like other parvoviruses that are able to replicate without the presence of a



13.9. Electron micrograph of purified goose parvovirus. A. Purified virions. B. Virions in the feces of a naturally infected 10-day-old gosling, showing intact and hollow (arrow) particles.

helper virus, GPV is dependent on cells actively synthesizing DNA for its replication cycle (44). It has been demonstrated that the replication of GPV under *in vitro* conditions is enhanced in the presence of a human helper-dependant adeno-associated type 2 virus (62).

Susceptibility to Chemical and Physical Agents

Goose parvovirus is very resistant to chemical and physical inactivation. Gough *et al*. (29) reported no loss of titer when the virus was heated at 65°C for 30 min. These authors also found that the virus was stable at pH 3.0 for 1hr at 37ºC. Schettler (75) tested an isolate against a variety of chemicals under different conditions and detected no significant loss of activity. However, following treatment with 0.5% formaldehyde infectivity was completely destroyed (75).

Strain Classification

The results of early studies using cross-neutralisation and gosling-protection tests suggested that several serologically distinct strains of the virus existed (20). However, at the time of these studies the etiology of the disease had not been confirmed; later work showed that several of the virus strains used were contaminated with reoviruses (21). Subsequent studies showed that parvoviruses from both geese and Muscovy ducks were antigenically closely related (26, 31, 41). Later studies with Muscovy duck isolates using cross neutralization, restriction endonuclease and molecular analysis identified significant differences between the genomes of MDPV and GPV (7, 9, 36, 82, 91). A more recent investigation described the isolation and characterization of a parvovirus from Muscovy ducks in Pennsylvania, USA associated with between 10 and 40% mortality. This isolate showed only about 85% identity with other goose and Muscovy duck parvoviruses in a conserved region of the genome, compared to over 99% and 95% identity among previously sequenced MDPV and GPV isolates (69)

Laboratory Host Systems

Goose parvovirus has only been isolated in embryonated goose or Muscovy duck eggs or primary cell cultures prepared from the embryos. It is essential that the embryonated eggs are obtained from unvaccinated flocks known to be free of parvovirus antibodies. Isolated virus has been cultivated in an embryonic goose fibroblast cell line (CGBQ).

Pathobiology and Epidemiology

Incidence and Distribution

Waterfowl parvoviruses have been reported from all the major goose and Muscovy duck farming countries of Europe, including

the former USSR and Israel. The disease has also been reported from the People's Republic of China and several of its autonomous regions, Taiwan, Vietnam, Japan and the USA (69, 86). There have been unconfirmed reports of outbreaks in Muscovy ducks in South America. A disease with similar clinical and post mortem features has also been reported from Canada, although parvoviruses were not isolated (70).

Natural and Experimental Hosts

Geese, Muscovy ducks and some hybrid breeds are the only species in which natural clinical disease has been observed. All breeds of domestic geese are susceptible and the disease has also been reported to occur in Canada geese (*Branta canadensis*) and snow geese (*Chen hypoborea atlantica*) following accidental infection (74). In a serological survey in wild geese in Germany 48% of the bean geese (*Anser fabalis*) and white-fronted geese (*Anser albifrons*) tested had significant neutralising antibody to GPV (34). Other breeds of domestic poultry and ducks appear refractory to experimental infection (29,35).

Age of Host Commonly Affected

The disease is strictly age dependant; thus, 100% mortality may occur in goslings under 1 wk of age, with negligible losses occurring in 4- to 5-wk-old birds. However, while older geese do not show clinical signs of infection, they respond immunologically (19, 26, 43). Similar findings apply to the clinical disease in Muscovy ducks (35, 53, 94).

Transmission, Carriers, Vectors

Infected birds excrete large amounts of virus in their faeces resulting in a rapid spread of infection by direct and indirect contact. The most serious outbreaks occur in susceptible goslings following vertical transmission of the virus. In older birds that become subclinically infected, a latent infection may become established. These birds may then act as carriers of the disease and transmit the virus through their eggs to susceptible goslings or ducklings in the hatchery (16, 46). No biological vectors have been identified.

Incubation Period

In susceptible goslings the incubation period is age dependent. Experimental infection of day-old goslings with GPV results in the appearance of clinical signs 3–5 days later. In 2- to 3-wk-old birds the incubation period may vary between 5 and 10 days (46, 73).

Clinical Signs

The clinical signs in susceptible goslings and ducklings also vary according to the age of the birds. In birds under 1 wk of age the course of the disease may be very rapid with anorexia, prostration, and death occurring within 2–5 days. In older birds, or those with variable levels of maternally derived antibody, the disease follows a more protracted course with the appearance of characteristic clinical signs. Initially, affected birds exhibit anorexia, polydipsia, and weakness with a reluctance to move. There is a nasal and ocular discharge in many birds with associated headshaking. The uropygial glands and eyelids are often red and swollen, and a profuse white diarrhoea is evident in many birds. Examination of the birds at this stage may reveal a fibrinous pseudomembrane covering the tongue and oral cavity. Survivors from the acute phase may develop a more prolonged disease characterised by profound growth retardation, loss of down around the back and neck, and marked reddening of the exposed skin. There may be an accumulation of ascitic fluid in the abdomen, which causes goslings to stand in a "penguin-like" posture.

In a comparative pathological study in domestic goslings and Muscovy ducklings strains of GPV caused severe disease in both goslings and Muscovy ducklings, whereas a strain of MDPV caused no clinical signs or pathological lesions in inoculated goslings (24).

Morbidity and Mortality

Mortality sometimes reaches 100% in goslings infected in the hatcheries. In 2- to 3-wk-old birds mortality levels may be below 10% although morbidity levels may be high. Complicating factors such as poor management and secondary bacterial, fungal, or viral infections may influence the final mortality levels (46, 52). Goslings over 4 wk of age rarely show clinical signs although a "late form" of the disease has been described in goslings 1–3 months of age (12). Geese and Muscovy ducks of all ages respond immunologically to parvovirus infection without necessarily showing clinical signs (43).

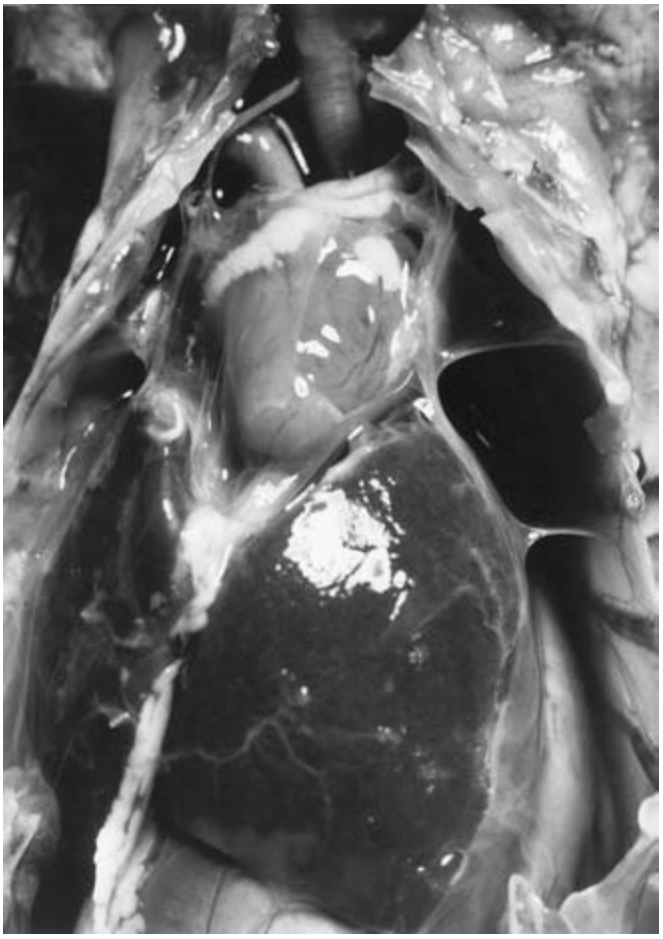
Pathology

Gross Lesions

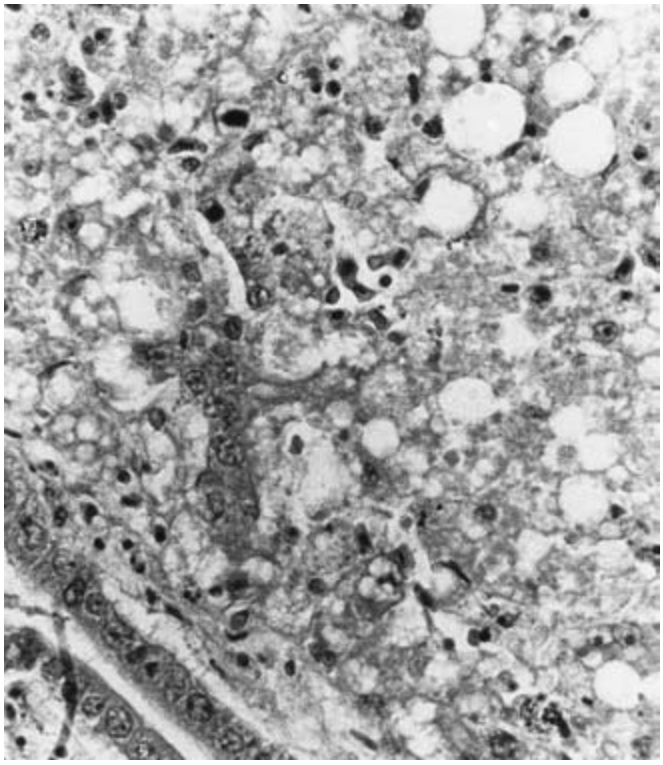
In acute cases of parvovirus infection, with a short clinical course, lesions are commonly found in the heart, which has a pale myocardium characteristically rounded at its apex (Fig. 13.10). The liver, spleen, and pancreas may be swollen and congested (16). A variety of other gross lesions may also be present in cases with a more prolonged clinical course. Typically, a serofibrinous perihepatitis and pericarditis are present with large volumes of straw-coloured fluid in the abdominal cavity. Pulmonary edema, liver dystrophy, and catarrhal enteritis may also be present. Less frequently, haemorrhages in the thigh and pectoral muscles may be seen. Diptheritic and ulcerative lesions may be observed in the mouth, pharynx, and esophagus, depending on the presence of secondary invaders.

Microscopic

Detailed histopathology studies of both GPV and MDPV infection by a number of workers have produced similar findings (11, 63, 65, 66). The main lesions reported were pronounced degenerative changes in myocardial cells with associated loss of striation, fatty infiltration (Fig. 13.11), and the presence of scattered Cowdry type-A intranuclear inclusions. Similar histological changes were also found in intestinal and smooth muscle cells. In liver the predominant lesions were degeneration of hepatocytes with vacuolation and fatty infiltration. Small, eosinophilic inclusion-like bodies were sometimes seen in the cytoplasm of the vacuolated hepatocytes. Changes in the pancreas consisted of shrunken, necrotic acinar cells with fatty infiltration. Some lym-



13.10. Postmortem appearance of a 12-day-old gosling infected with goose parvovirus showing hydropericardium and ascites. The liver is coated with a fibrinous membrane.



13.11. Liver section from a 10-day-old gosling infected with goose parvovirus showing widespread vacuolation and degeneration of hepatocytes.

phoblastic processes were occasionally observed in the spleen, bursa of Fabricius, and thymus, together with marked vacuolation of the kidneys. In Muscovy ducklings muscle fiber degeneration, mild sciatic neuritis and polioencephalomyelitis has also been reported (24). The pathological features will vary depending on the age of the birds at the time of infection.

Pathogenesis of the Infection Process

Detailed studies on the pathogenesis of waterfowl parvovirus are not available. In horizontal transmission, ingestion of virus-contaminated feed and water is of prime importance (46). Initially, virus replication occurs in the intestinal wall and then enters the blood stream. Following a viremia, virus reaches the liver and heart where the most severe pathological changes occur (46). It has been suggested that an enteric form of GPV exists (24).

Immunity

Passive Immunity

Adult breeding geese and Muscovy ducks that have been naturally infected with parvovirus, either during the rearing phase or as adults, transfer maternal antibody of the IgG type via the egg yolk to their progeny (18, 27, 35). This passively acquired antibody may persist at a relatively high level until about 2wk of age (43).

Humoral Immunity

The primary humoral response of geese to parvovirus infection is characterised by the initial production of IgM and then IgG-type immunoglobulin (43). Using virus-neutralisation (VN) and agar-gel precipitin tests to measure parvovirus antibodies in the sera of geese that had survived the disease, high and persistent levels of antibody were detected for up to 80 months after infection. The progeny of these geese were also found to be fully resistant to experimental challenge up to 4 wk of age (27). The results of studies by Kisary (43) suggested that goslings are not fully immunocompetent until 20 days of age.

Active Immunity

Cell Mediated Immunity

Cell mediated immunity is not thought to play a significant role in immunity to waterfowl parvovirus.

Diagnosis

Isolation and Identification of the Causative Agent

Waterfowl parvovirus can be isolated from a variety of suitable postmortem specimens following inoculation of 10- to 15-day-

old embryonated goose or Muscovy duck eggs via the allantoic cavity. Embryo mortality occurs 5–10 days post inoculation with haemorrhages and ochre-colored livers. The virus can also be isolated in primary cell cultures of goose or Muscovy duck embryos. Isolation of the virus is facilitated by inoculating cultures before they reach confluency (49). The virus produces a well-defined cytopathic effect (CPE) 3–5 days postinfection, although several blind passages may be required before a detectable CPE is observed, particularly when attempting to isolate MDPV. In haemotoxylin-eosin stained infected cultures Cowdrey type-A intranuclear inclusions and syncytium formation are often present (49, 77). The presence of the virus can be confirmed by electron microscopic examination of infected cell cultures, even though gross cytopathic changes are absent, or neutralisation with specific parvovirus antiserum (28).

Direct Detection of Viral Antigen

Immunofluorescence has been used to detect the presence of viral antigen in goslings (1), embryonated goose eggs (85) and infected cell cultures (75). Other methods have also been developed, including immunoperoxidase techniques (71), antigen-capture enzyme-linked immunosorbent assays (ELISA) (40) and reverse indirect haemagglutination test (87). A digoxigenin-labelled DNA probe technique has been described for the detection and identification of MDPV (57).

An agar gel diffusion technique has been described using rabbit anti-goose parvovirus serum to precipitate parvovirus in the allantoic fluid of infected goose embryos (5).

Electron Microscopy

Goose parvovirus virions have been detected by EM in the faeces of goslings showing clinical signs of goose parvovirus (25) and in ultrathin sections of heart and bursa of Fabricius from infected goslings (4). An immune EM (IEM) technique has also been developed and evaluated for the detection of parvovirus. The technique, utilizing GPV monoclonal antibodies, detected parvovirus virions in the organs of goslings and cells of infected goose and duck embryos (1)

Molecular Identification

Various polymerase chain reaction (PCR) techniques have been developed to detect both GPV and MDPV. Primers have been designed from conserved regions of the VPI, 2 and 3 genes that encode for the capsid proteins, and can be used to differentiate strains of GPV and MDPV following sequencing and restriction fragment length polymorphism (RFLP) (69, 76, 80, 82, 91). A PCR detected goose parvovirus DNA in a range of tissues from experimentally infected ducklings from 2 days post inoculation (58). Nucleic acid dot-blotting techniques have also been developed to detect the presence of GPV DNA in a range of tissues from infected geese (89).

Serology

Serologic tests are useful in evaluating the immune status of breeding flocks of geese and Muscovy ducks and their progeny. The presence or absence of parvovirus antibody in breeder geese will determine the susceptibility of the progeny. Serology is also a useful diagnostic tool in confirming recent outbreaks of the disease in goslings and Muscovy ducklings. Demonstration of yolk-derived antibody in eggs will also provide information on the levels of maternal-derived antibody in the offspring.

Virus Neutralisation

The most widely used method is the VN test in embryonated goose or Muscovy duck eggs or primary cell cultures, to detect the presence of GPV-neutralizing antibodies (28). Cross neutralisation tests can be used to differentiate between GPV and MDPV antibodies (3). A duck embryo-adapted GPV strain has also been used for VN tests in Pekin duck embryonated eggs (30). Titres of 1/16 or greater are considered positive for GPV antibodies.

Agar Gel Precipitation

Though less sensitive than the VN test the agar gel precipitation (AGP) test is a useful method for testing large numbers of sera for the presence of parvovirus antibodies. The test does not differentiate between antibodies produced against parvovirus derived from geese or Muscovy ducks (26, 60).

Other Serologic Tests

Enzyme-linked immunosorbent assays (ELISA) have been developed to detect goose parvovirus antibodies in both geese and Muscovy ducks (33, 36, 55). A blocking ELISA, incorporating anti-goose parvovirus-goose IgG, has been developed and compared to other serologic tests (40). The results showed that the method was rapid, reliable, easily standardised, and correlated well with a virus-neutralisation test. This type of ELISA has also been used to detect MDPV antibodies and a good correlation was reported between ELISA values and protection to challenge (40). Problems may arise from this type of test if non-specific-pathogen-free geese are used in the production of serum and immunoglobulin G for use in the ELISA. Other serological techniques that have been developed include the spermagglutination-inhibition test (61), a plaque reduction assay (78) and indirect immunofluorescence (81). A Western blotting assay on sera from infected goslings and Muscovy ducklings using purified antigens from the capsid and non-structural proteins of waterfowl parvoviruses has also been described (84).

Differential Diagnosis

Formerly, parvoviruses originating from geese and Muscovy ducks were thought to be antigenically closely related. However, following the emergence of a more virulent Muscovy duck strain of the virus, studies using VN, restriction endonuclease analysis and molecular sequencing of the virus genome have demonstrated significant differences between GPV and MDPV isolates (7, 28, 69).

A digoxigenin-labelled DNA probe has been developed for the detection of MDPV. The assay proved sensitive for detecting strains of parvovirus and differentiating isolates from vaccine-derived strains (57).

Avian adenovirus-associated viruses are defective par-

voviruses that may be associated with adenovirus infections. In the absence of a helper adenovirus these parvoviruses are unable to replicate under *in vitro* conditions.

Very few other pathogens of goslings and Muscovy ducklings exist that show the strict age-relatedness of waterfowl parvoviruses. The herpesvirus of duck viral enteritis produces disease with high mortality in geese and ducks of all ages. Isolation and identification of the causal virus will clearly differentiate it from parvoviruses. Duck hepatitis viruses also cause fatal diseases in ducks under 6 wk of age. However, these viruses are not pathogenic for geese or Muscovy ducks.

Hemorrhagic nephritis and enteritis of geese (HNEG) affects geese from 4 to 20 wk of age. The disease was first reported from particular regions of France in the 1970's and was referred to as the late form of Derzsy's disease (47). More recent studies suggest that HNEG is associated with an avian polyomavirus (28). A disease of 2–8-wk-old Muscovy ducklings called virus K disease of the Muscovy duck has been described (23). The etiological agent is thought to be a reovirus and it does not cause disease in geese.

Pasteurella anatipestifer and *Pasteurella multocida* organsims may also cause high mortality in goslings and Muscovy ducklings. Treatment of birds with appropriate antibiotics and culture of the etiologic agent in suitable media will enable differentiation from waterfowl parvovirus.

Intervention Strategies

Management Procedures

Because many outbreaks of waterfowl parvovirus are directly attributed to transmission of the disease by congenitally infected birds during hatching, the practice of incubating and hatching eggs that have originated from different breeding flocks should be discouraged. Only eggs from known parvovirus-free flocks should be incubated together and good hatchery hygiene should be maintained.

On farms where outbreaks of the disease have occurred, the practice of breeding from parent stock that have survived the disease when young should also be discouraged, as these birds are potential carriers of the virus. All contact birds, whether young or adults, should be serologically tested in order to identify which birds have been infected horizontally. Positive reactors should be removed from the flock as these birds may also become carriers of the virus.

Vaccination Strategies

Because the disease is confined to young geese or Muscovy ducklings, control measures have been developed to provide adequate immunity during the first 4–5wk of life. Some of the early outbreaks of GPV occurring in China in 1962 were controlled by the use of hyperimmune serum in newly hatched goslings (22). Serum therapy was widely used when the disease subsequently appeared in Europe, using serum produced in hyperimmunized geese (16, 32, 35, 72). However, passive immunization was found to be expensive and time consuming, particularly as two doses of serum were often required to produce adequate immunity (50). Active immunization of adult breeding geese and Muscovy ducks with virulent virus has also been reported (35). The results showed that good protection was transferred to the progeny via the egg yolk.

One of the first vaccines against GPV was developed in China, and during the period 1962–79 about four million female geese were vaccinated (22). The virus was attenuated following multiple passages in embryonated goose eggs and good protection to challenge was recorded in the progeny goslings. Other vaccines have been developed by attentuation of the virus in goose or Muscovy duck embryo cell cultures, for use in breeding geese and goslings (39, 50, 59, 88, 93). Duck embryo-adapted GPV vaccines have also been shown to induce a good immune response in goslings and breeder geese (8, 30).

Inactivated vaccines have also been used in flocks of breeding geese and Muscovy ducks inducing high levels of immunity (37,79). Bivalent vaccines have also been developed containing both GPV and MDPV antigens.

Future Developments

Recombinant vaccines formulated in oil emulsion have been evaluated in goose and Muscovy duck laying flocks. The authors report that the progeny of the vaccinated breeders were fully protected against goose parvovirus (48).

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Chapter 14

Other Viral Infections

Introduction

Y. M. Saif

This chapter has traditionally included viral infections that did not fit into virus groupings used in a given edition. Yet, the grouping of viral infections into different chapters has changed in the different editions for a variety of reasons. Avian encephalomyelitis is presented in this edition as a subchapter instead of its earlier status as a stand-alone chapter in recognition of its rare occurrence in commercial poultry. Hepatitis E infections are presented as a completely new subchapter replacing two earlier subchapters, "Big Liver and Spleen Disease," and "Hepatitis Splenomegaly Syndrome." These two subchapters were included in Chapter 34, "Emerging Diseases of Complex or Unknown Etiology," of the 11th edition. Consensus now is that these two conditions are the same clinical enteritis. In addition, the work of Meng *et al.* has elucidated the etiology of the condition being a hepatitis E virus.

The etiology of proventriculitis in chickens continues to be elusive, and in this edition, it is presented in Chapter 33, "Emerging Diseases of Complex or Unknown Etiology."

Miscellaneous Herpesvirus Infections

J.P. Duchatel and H. Vindevogel

Introduction

Herpesvirus infections have been described in several species of domestic and wild birds including pigeons (10, 38), psittacine birds (32), falcons (24), owls (4), cormorants (13), cranes (5), storks (19), and bobwhite quail (18).

All herpesvirus strains isolated from pigeons in Belgium, France, Australia and Czechoslovakia have been found to be antigenically similar and to possess the same cultural characteristics (3, 17, 21, 38, 39). Therefore, only one pigeon herpesvirus type, pigeon herpesvirus 1 (PHV1) appears to exist. However, following Kaleta (17), various breeds of racing and show pigeons may harbor two serologically different herpesviruses and some isolates may contain small- and large-plaque variants.

PHV1 is antigenically different from turkey herpesvirus, Marek's disease virus, infectious laryngotracheitis virus, and duck virus enteritis herpesvirus (25, 27). It can also be clearly distinguished from the psittacine herpesvirus (Pacheco's disease virus) based on antigenic composition and plaque size in cell culture (49).

Conversely, PHV1 cannot be serologically distinguished from the falcon (FHV) and the owl (OHV) herpesviruses, and it remains to be established whether these three herpesviruses are different isolates of the same virus (24, 25). All other herpesviruses isolated from wild birds differ antigenically from each other and from other avian herpesviruses except for the bobwhite quail herpesvirus and the crane herpesvirus, which are serologically related (18).

This subchapter will cover PHV1 infections in pigeons and will briefly mention pseudorabies virus infections that can be experimentally induced in pigeons and young chickens.

History

The first observation of intranuclear inclusion bodies in the liver of pigeons, probably associated with PHV1 infection, was reported in 1945 (33). Since 1967, PHV1 has been isolated from diseased pigeons in numerous countries (9, 38).

Incidence and Distribution

The suspected geographic distribution of PHV1 is worldwide. The virus has been isolated in the United Kingdom (8), Czechoslovakia (21), Australia (3), Belgium (43), Hungary (37), Germany (14), France (23) and Italy (56). Infection has also been observed in the United States (27). In Europe, the vast majority of pigeons are infected, since more than 50% of them possess specific antibodies (16, 22, 38, 50). In Belgium, the presence of PHV1 was demonstrated in 60% of dove-cotes where pigeons

Contributions of Dr. Hitoshi Kawamura to previous chapters are gratefully acknowledged.

were permanently affected with respiratory disease, and PHV1 could be isolated from the pharynx of 82% of pigeons affected with acute coryza (38, 51).

Etiology

Belonging to the family of *Herpesviridae* (most probably alpha), PHV1 is called *Columbid herpesvirus 1* in the new nomenclature. It possesses the morphology and has the physico-chemical properties of a typical herpesvirus (58).

All avian cell cultures tested to date were susceptible to PHV1, but the cytopathic effects varied (7,44,45). In chicken embryo fibroblast (CEF) cultures, the most consistent change is an increase in the size of cells with syncytia containing two to four nuclei. Initial alterations consist of margination of chromatin and the appearance of Cowdry type-A intranuclear inclusion bodies 10 hr after inoculation. Viral antigen is first detected in the nucleus and later throughout the cytoplasm. Virus can be detected by 12 hr, and peak titers are reached by 36 hr after inoculation (44). The baby hamster kidney cell line (BHK) is also susceptible to infection with PHV1, but all the other mammalian cell lines tested so far have been refractory to the virus (45).

Plaques develop in cultures overlaid with carboxymethylcellulose, agarose, or specific antiserum (42, 43). Virus multiplication in cell cultures is inhibited by trisodium phosphonoformate (28, 29, 38) and acycloguanosine (35). Extracellular virus can be protected by the addition of 5% dimethylsulfoxide to the medium before freezing (43).

Pathogenesis and Epidemiology

Natural and Experimental Hosts

The pigeons seem to be the natural host of PHV1 with virus infection remaining latent (38). PHV1 has been isolated from budgerigars (*Nymphicus hollandicus*) accidentally infected after close contact with pigeons (46). Pigeons are susceptible to experimental infection by pharyngeal painting, which causes a mainly localized disease (42, 48), or by the intraperitoneal route, causing a systemic infection (10). Systemic infections can also be produced in budgerigars (*Melopsittacus undulates*) by intranasal inoculation of the virus (39). Chickens, ducks, canaries and hamsters are resistant to infection (10, 38, 40).

Transmission

Susceptible pigeons can be infected through direct contact with infected birds. Egg transmission of PHV1 seems unlikely (41). Mature pigeons in infected flocks are asymptomatic carriers of the virus, and some of them may shed virus from time to time (48).

The vast majority of latently infected mature pigeons re-excrete virus in their throat during the breeding season and during squab gorging (60). They are, therefore, able to transmit directly the infection to the squabs soon after hatching. Although the squabs become infected, they are protected from the disease by maternal antibodies acquired through the egg yolk. Therefore, most of the squabs themselves become asymptomatic carriers after this initial infection (41). The virus has been isolated by us from the cloacal bursae and the kidneys of 12-day-old pigeons.

Incubation Period, Shedding, and Latency

Virus excretion begins 24 hr after inoculation and persists at a high titer for a minimum of 7–10 days in inoculated squabs. Typical lesions appear 1–3 days after infection when viral excretion reaches its peak. Mild episodes of recurrence, without clinical signs, occur spontaneously. High titers of specific antibodies do not prevent these recurrences, and, conversely, recurrent episodes are not more frequent when the animals are nearly devoid of specific antibodies. Pigeon herpesvirus 1 re-excretion can also be provoked by cyclophosphamide (Cy)-treatment of pigeons and this period of re-excretion may be accompanied by lesions (48).

Distribution of Pigeon Herpesvirus 1 in the Host

In classic PHV1 infection, virus generally remains localized in the upper respiratory and digestive tracts. However, naturally occurring experimental pharyngeal infection may be followed by viral dissemination throughout the body, with viral localization and development of lesions in organs such as trachea, spleen, liver, kidney, and brain (6, 8, 41, 42). Indeed, during the primary infection and during episodes of re-excretion following Cy-treatment, a transient viremia may occur (42). Moreover, PHV1 can be transmitted from cell to cell in the presence of high titers of specific antibodies (42). Thus, PHV1 can be spread either by tissue contiguity or by viremia, especially when pigeons are immunodepressed (38).

As about 65% of racing pigeons are infected with circovirus (11, 12, 34) and suffering from immunodeficiency, PHV1 is now a major viral infection associated with high morbidity. PHV1 DNA has been detected in 7/45 liver samples from pigeons with symptoms of circovirus infection from 5 of 15 lofts (26).

Signs

In the acute form of the disease, pigeons sneeze frequently and show conjunctivitis, and nostrils become obstructed with nasal mucus and moisture. Caruncles, which are normally white, turn yellow-gray.

In the chronic form, sinusitis and intense dyspnea may be observed if the primary viral infection is complicated by *Trichomonas columbae* or secondary mycoplasmal or bacterial invaders (*Mycoplasma columbinum, Mycoplasma columborale, Pasteurella multocida, Pasteurella hemolytica, Escherichia coli, Staphylococcus ß-hemolysin, Streptococcus ß-hemolytic)* (30, 38).

Morbidity and Mortality

Clinical disease is observed principally following primary infection of young pigeons not protected by maternal antibodies and in virus carriers in which the infection is complicated by virtue of debilitating factors (41).

Pathology

Gross Lesions

The mucous membranes of the mouth, pharynx, and larynx are congested and, in severe cases, covered with foci of necrosis and small ulcers. The mucous membrane of the pharynx may be coated with diphteritic membranes. When the viral infection is

generalized (viremia), foci of necrosis can be observed in the liver. If the initial infection becomes complicated by bacterial infections, the trachea may be obstructed by caseous material and some birds may show airsacculitis and pericarditis (pigeons chronic respiratory disease) (38).

Histopathology

Multiple foci of necrosis are observed in the pharyngeal stratified squamous epithelium and in the salivary glands. Foci contain cells at different stages of degeneration and necrosis, and intranuclear inclusions are present in adjacent epithelial cells. Large foci may extend and form ulcers. Similar foci of necrosis can also be observed in the laryngeal and tracheal epithelium (42).

In generalized infections, pigeons presenting with hepatitis consistently have intranuclear inclusion bodies in many hepatic cells widely spread throughout the organ (8, 42, 43). Lesions have also been described in the pancreas and the brain (6, 8, 10).

Immunity

Neutralizing antibodies appear in squabs at the end of the first week following infection. Importance of these antibodies is difficult to evaluate with regard to re-emergence of active infection (48), but when acquired passively in the form of maternally derived antibodies, they are protective for squabs (41). As a herpesvirus infection, it might be presumed that cell-mediated immunity is important in PHV1 infections.

Diagnosis

Isolation and Identification of Causative Agent

Pigeon herpesvirus 1 can be easily isolated in CEF cultures from pharyngeal swabs of infected pigeons ; also, but with more difficulty, from internal organs such as trachea, lungs, or liver. Isolates should be characterized by immunological means such as immunofluorescence (38, 47).

A PHV1 polymerase chain reaction (PCR) amplifying a 242 base pair product of the DNA-dependent DNA polymerase gene has been described (26).

Serology

Specific antibodies can be titrated by virus-neutralization tests or by indirect immunofluorescence and can be detected by counter-immuno-electro-osmophoresis (38, 47).

Differential Diagnosis

Clinically acute PHV1 infection disease may be confused with Newcastle disease virus infection (lentogenic pneumotropic paramyxovirus 1 strains), and chronic PHV1 infection complicated by secondary bacterial invaders must be distinguished from the diphteroid form of poxvirus infection (57, 59). A diagnosis of PHV1 infection requires virus isolation or serological evidence; however, both techniques may fail to demonstrate PHV1 infection in individual pigeons, the first because the animal may not be actively excreting virus and the second because of an absence of seroconversion in a latent carrier. For these reasons, several animals from the same dove-cote must be simultaneously examined (38, 47).

Treatment, Prevention, and Control

After primary infection, pigeons become asymptomatic carriers and may re-excrete virus. Chemotherapy trials with trisodium phosphonoformate and acycloguanosine failed to prevent infection (28, 29, 35, 52). Vindevogel *et al.* (53, 54, 55), therefore, compared the ability of inactivated (in oil adjuvant), or attenuated vaccines to prevent clinical disease, the carrier state, and virus re-excretion. Both types of vaccine were able to reduce primary viral excretion and clinical signs of challenge. Nevertheless, neither attenuated nor inactivated vaccines were able to prevent appearance of carriers since most of the pigeons re-excreted virus after Cy-treatment. Vaccination did, however, help to prevent viral re-excretion, thereby helping to control viral dissemination.

Pseudorabies Infection of Birds

Pseudorabies virus [*Sus herpesvirus 1* (SHV1)] produces a generally mild disease in swine, its natural host, but a fatal disease in cattle. Other animals found infected in nature are dogs, cats, sheep and rats (20, 23). The virus multiplies very well in chicken embryo fibroblast cultures (2).

Experimentally, SHV 1 can infect chickens, chicken embryos, and pigeons (15, 20, 36). Chicken embryos succumb with encephalitis after inoculation on the chorioallantoic membrane, as do 2-day-old chicks after being inoculated subcutaneously (1). Adult chickens, however, are resistant to subcutaneous inoculation (31).

Toneva (36) attenuated a strain of SHV 1 by serial passages in pigeons combining intramuscular and subcutaneous routes of inoculation (pigeon strain 80). Inoculated pigeons developed classic symptoms of encephalitis, i.e. torticollis, and disordered balance. The SHV 1 pigeon strain 80 is avirulent for rabbits, mice, guinea pigs and piglets after subcutaneous injection but remains lethal after intracerebral inoculation.

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Avian Nephritis

Tadao Imada

Introduction

Avian nephritis, caused by an astrovirus, is an acute, highly contagious, typically subclinical disease of young chickens that produces lesions in the kidneys.

The causative agent, avian nephritis virus (ANV), was first isolated in chicken kidney cell (CKC) cultures from the rectal contents of apparently normal, 1-wk-old broiler chickens in Japan in 1976 (46). It has been shown to be an astrovirus, which is distinct from avian encephalomyelitis virus, duck hepatitis viruses, and turkey astrovirus, based on pathologic (9, 18), immunologic (3, 22, 23, 27, 42), and genomic (14, 15,20) criteria. ANV is the first astrovirus that was not identified by the morphological but by genomic analyses (14). The pathogenicity of this virus was established by experimental infection of chickens and chicken embryos. As the isolation and identification of this virus was a little bit difficult, there have been a few reports on the disease associated with this virus infection in the field (19, 36, 42), the economic importance is not well known, and public health significance is not known. Lately, more information about avian astroviruses has been published (1, 16, 17).

Incidence and Distribution

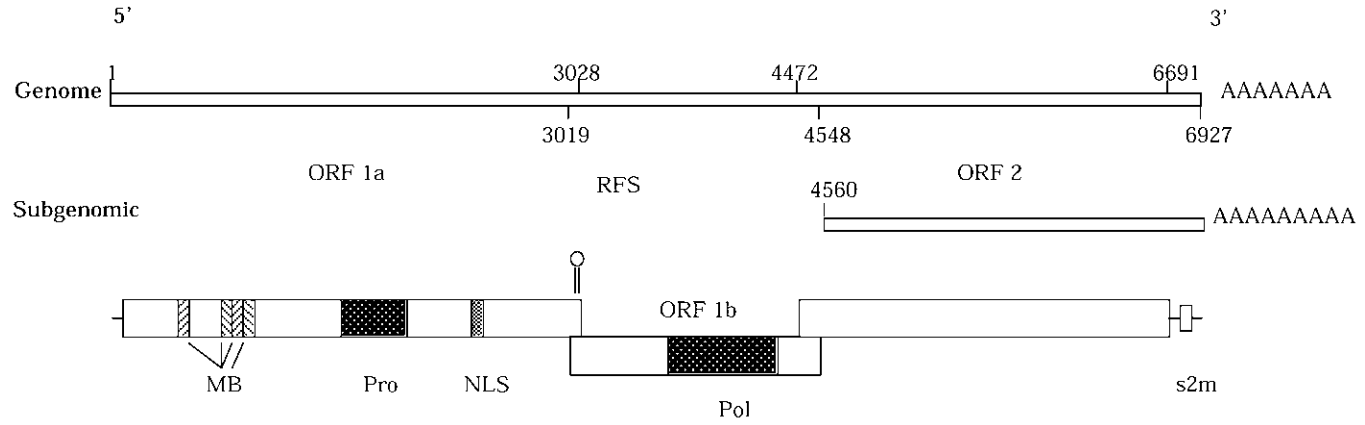
The true incidence and distribution of the disease are not well known owing to the transient, usually subclinical nature of the infection and the difficulties with virus isolation. Runting and diarrhea in young chickens associated with an enteroviruslike particle serologically identical or related to ANV, but different biologically, have been reported in some countries (4, 6, 7, 8, 22, 24, 25, 33, 40). Avian nephritis virus has been shown to be widely distributed in chicken flocks in Japan (10, 43, 45), some European countries (3, 5, 32), and several specific-pathogen-free (SPF) flocks (3, 26, 32) by serology. Antibody to ANV has been also detected in turkeys in Northern Ireland and England (3, 32). Recently, the ANV gene has been validated on the case of acute nephritis and gout in Hungary (19).

Etiology

Classification and Morphology

Avian nephritis virus is classified as a new genus member of the family *Astroviridae* based on the following properties: 1) the ANV genome consists of approximately 7,000 nt with three Open Reading Frames (ORFs), ORF 1a, 1b, and 2 (Fig. 14.1). ORF 1a encoded a 3C-like serine protease motif, whereas the ORF 1b encoded a viral RNA-dependent RNA polymerase motif, and a ribosomal frameshift motif was also present between the two ORFs. On the other hand, ORF 2 of ANV, which may encode the capsid precursor polyprotein, encoded a product with 26% amino acid homology to those of human astrovirus (HAst). As shown for the HAst, ORF 2 of ANV is likely expressed from subgenomic-size RNA, which was detected in the ANV-infected cells, as well as genomic-size RNA. The genome organization of

Contributions of Dr. Hitoshi Kawamura to previous chapters are gratefully acknowledged.



14.1. Schematic representation of the ANV (G-4260 strain) genome. Open boxes, PRFs. The locations of three ORFs, predicted transmembrane helices (MB), protease (Pro), nuclear localization signal (NLS), ribosomal frameshift structure (RFS), RNA-dependent RNA polymerase (Pol), and stem-loop II-like motif (s2m) are indicated. Numbering is according to the ANV genomic sequence (accession no. AB033998).

ANV is apparently identical to that of HAst; 2) ANV differs from previously described mammalian astroviruses in that trypsin is not required for growth in tissue culture. Trypsin-dependent replication of astroviruses *in vitro* is a very specific feature; 3) ANV was genetically distinct from other astroviruses; 4) replication in the cytoplasm; 5) diameter of 28 nm; 6) resistance to ethyl ether, chloroform, trypsin, and acid (pH 3.0); 7) relative heat lability; and 8) partial stabilization at 50°C by molar magnesium chloride (2, 14, 21, 46).

It is quite difficult to differentiate this virus from small round viruses, especially from picornavirus morphologically (16, 21).

Laboratory Host Systems

Chicken Embryos

When inoculated with ANV by the yolk sac route, 6-day-old embryos died 3–14 days postinoculation (PI). They manifested hemorrhage and edema of the whole body at 3–6 days PI and stunting at 7–14 days PI. When inoculated by the chorioallantoic membrane (CAM) route, high virus doses killed all embryos, but low virus doses allowed some infected embryos to hatch normally. In these eggs, the CAM showed edematous thickening or pocks at the inoculation site, and the embryos were stunted. Embryos inoculated by the allantoic cavity route sometimes became infected, but no virus was detected in allantoic fluids (8, 12).

Cell Culture

The representative strain (G-4260) of ANV grew in CKC with round cell-type cytopathic effect (CPE) and maximum virus titers at 24 hr PI (46). It did not grow in duck embryo fibroblasts, duck embryo kidney cells, or some established mammalian cell lines (HeLa, Vero, MDBK, PK-15, and MDCK). But the ability of ANV to replicate and to show CPE *in vitro* may be influenced by the conditions of the cell cultures and the strains of ANV (3, 6, 8, 27, 42). It has been shown that the chick embryo liver cells cultures and LMH cells, a chicken heptocellular carcinoma cell line, are suitable for chicken astroviruses isolation and propagation (1).

Pathogenicity

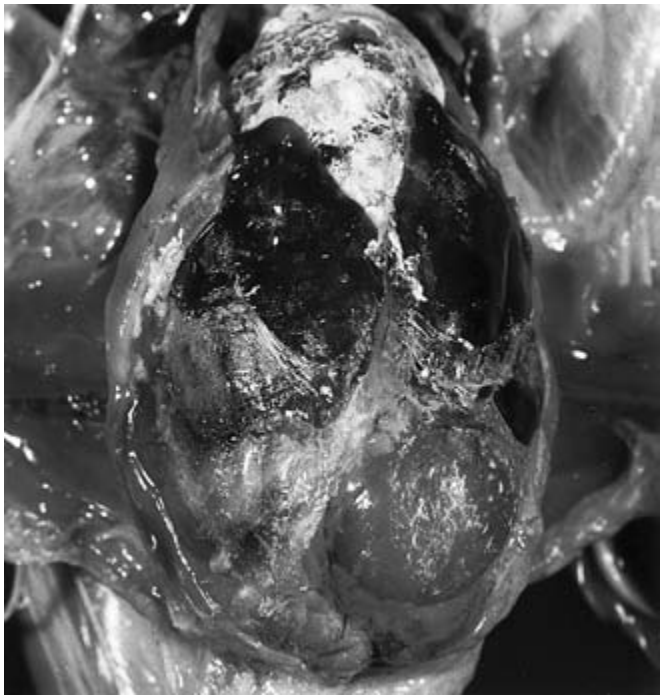
Young chickens are the only animals known to develop clinical disease and distinct kidney lesions when exposed to ANV. Field viruses exhibit different degrees of pathogenicity in chickens. There are indications that different serotypes, and even strains with the same serotype, can vary in their ability to produce illness and death (6, 8, 9, 19, 34, 38, 39, 40, 42). Avian nephritis virus had no apparent effect on egg production or egg quality in laying hens (13). It had been reported that infectious bursal disease virus infection and cyclophosphamide treatment enhanced the pathogenicity of ANV in chickens (30, 31).

Pathogenesis and Epidemiology

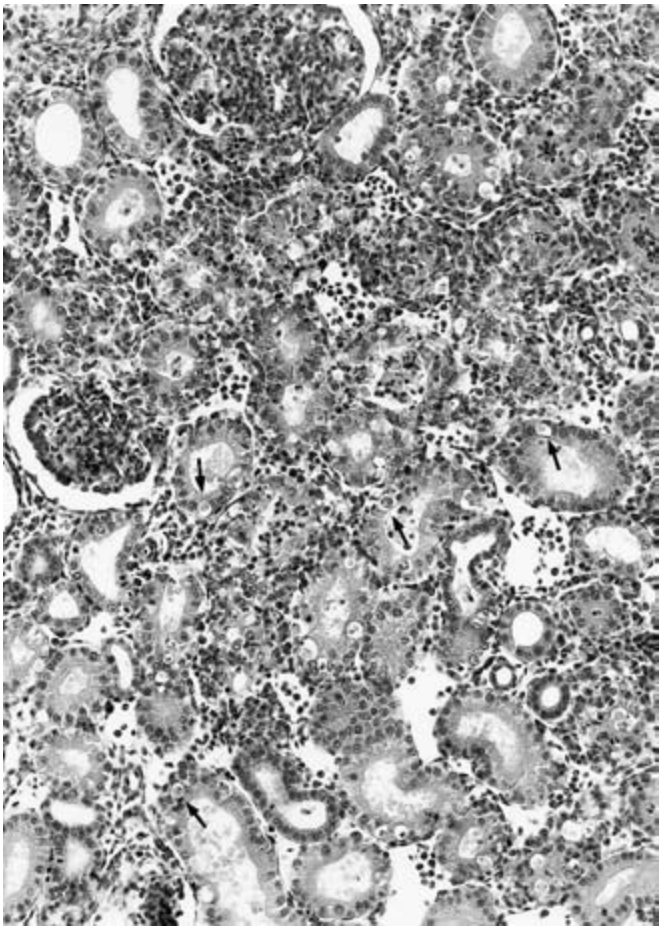
Natural and Experimental Hosts

Infection has been recognized in chickens, and antibodies to ANV were detected in turkey flocks. Attempts to establish active infection in other animals have not yet been carried out. Chickens of all ages may be infected, but it has been observed that 1-day-old chicks are the most susceptible (8, 11, 29). Transmission readily occurs by direct or indirect contact (11). Egg transmission has been suggested on the basis of field observations (3, 42), and the virus can be isolated from chicks hatched from artificially infected embryonating eggs (12). In experimentally infected chicks, the virus was first detected in feces 2 days PI, with maximum virus shedding at 4–5 days PI. The virus is widely distributed, with maximum titers in the kidney and jejunum and lower titers in the bursa of Fabricius, spleen, and liver. The virus was consistently isolated from kidney, jejunum, and cloaca, but not from brain and trachea during the first 10 days PI (9).

The only clinical sign of ANV infection in 1-day-old chicks is



14.2. Visceral urate deposits in a chick that died 10 days postinfection. Chalklike urate crystals were deposited on the surface of the peritoneum and liver, although those on the surface of the liver were mostly removed during necropsy. The heart is white due to heavy urate deposits on the epicardium.



14.3. Degenerated proximal convoluted tubules containing acidophilic granules (*arrows*) in epithelial cell cytoplasm, and lymphocytic infiltration in interstitium, 5 days postinfection. H & E, ×300.

transient diarrhea, but not all chicks show this sign. Weight gain is depressed between 7 and 14 days PI. At necropsy at 4–21 days PI, mild to severe discoloration and swelling in the kidneys are observed, and in dead chicks within 2 weeks PI, visceral urate deposits (Fig. 14.2) are observed (7, 9, 18, 28, 29, 34, 35, 37). It has been reported that the concentration of serum uric acid or plasma urate value of ANV-infected 1-day-old chicks is transiently higher than that of uninfected chicks (28, 29, 30, 31, 37, 39).

Mortality may be influenced by the virulence of the ANV strain, strain of birds, and experimental conditions (8, 34, 39, 44).

Under field conditions, clinical signs associated with this virus infection in broiler chickens have varied from none (subclinical) to outbreaks of the so-called runting syndrome and baby chick nephropathy (7, 10, 19, 22, 24, 36, 40, 42, 46). Nothing is known about clinical signs in turkeys.

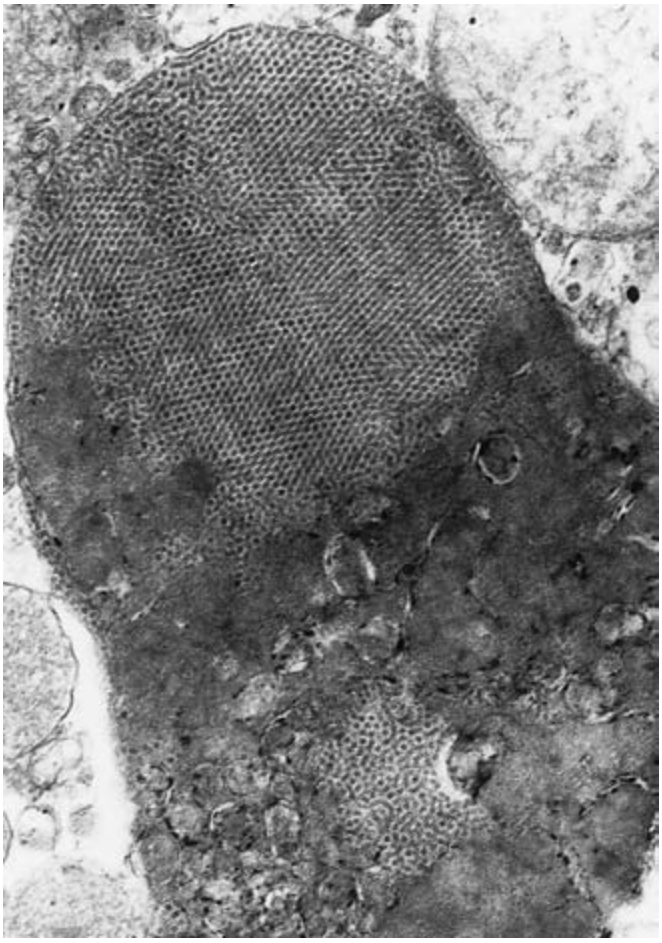
Histopathology

Histopathologic lesions in the kidneys of experimentally infected chickens have been studied (8, 11, 12, 13, 18, 28, 29, 30, 31, 34, 35, 37, 38, 40). The primary changes consisted of necrosis and degeneration of epithelial cells of the proximal convoluted tubules with infiltration of granulocytes. The degenerating epithelial cells had acidophilic granules of various sizes in the cytoplasm (Fig. 14.3). Also, there was interstitial lymphocyte infiltration and moderate fibrosis. In later stages, at 14–28 days PI, lymphoid follicles developed. Avian nephritis virus particles and viral antigens were demonstrated by electron microscopy in the degenerating epithelium (Fig. 14.4) and immunofluorescence (IF), respectively. Specific viral antigens were recognized by IF also in the jejunum, but distinct microscopic lesions were not observed in the small intestine. The chicks that died revealed many urate tophi in the serosa and parenchyma throughout the body, including the kidneys.

Diagnosis

Isolation and Identification of Causative Agent

For isolation of the virus from infected chickens, suspensions of either the kidneys or the rectal contents made in cell culture medium can be used as inoculum. After freezing and thawing three times, and centrifuging to remove the large tissue particles, the supernatant fluid is inoculated onto monolayers of CKCs or injected by the yolk sac route into 6-day-old embryonating eggs that originated from an SPF flock with no antibody to ANV (44,



14.4. Crystalline array of virus particles in the cytoplasm of a kidney epithelial cell, 3 days postinfection. ×30,000.

46). In infected CKC cultures, round cell-type CPE, without hemagglutinin, develops within 72 hr PI. There may be difficulties associated with isolation of enteric viruses in cell cultures (see Chapter 12, Diagnosis).

In embryonating eggs, infected embryos display hemorrhage and edema or stunting. Virus isolates may be further characterized by filtration through 50-nm-porosity membrane filters, or by inoculation of 1-day-old chicks with a 50% suspension of tissues harvested from embryos, followed by examination for lesions in the kidneys 3–7 days PI.

The IF technique is a useful diagnostic procedure, because this technique could detect group antigens of ANV. Viral antigens can be detected in the early acute phase of the disease by staining infected kidneys with specific anti-ANV fluorescent antibodies. This technique can also be used to detect viral antigens in cell cultures and embryos. In CKC infected with ANV, lumpy and granular antigens are seen in the cytoplasm as early as 12 hr PI.

For confirmation and direct demonstration of ANV infection, nucleic acid-based methods can be used. RT-PCR method could detect the regions of the viral protease, polymerase, and capsid genes specifically (1, 14, 19)(see Chapter 3, Diagnosis).

Serology

Chickens recovered from naturally occurring and experimental infections manifest an immunologic response that can be measured with a conventional virus-neutralization test, the indirect IF test, and enzyme-linked immunosorbent assay (ELISA) (5). It has been reported that there are at least two serotypes of ANV in the field (8, 38, 40, 42).

Differential Diagnosis

Certain nephrotoxic strains of infectious bronchitis virus (IBV) cause interstitial nephritis. It would be difficult to separate the two conditions on the basis of the histologic lesions (41). These cases may be differentiated from ANV infections by the fact that with infectious bronchitis there are some changes in the trachea, and infections in kidneys are usually preceded by respiratory signs. When nephritis is noticed in especially young chickens, it is necessary to isolate the causative agent or conduct serologic tests. The possibility that the two diseases may occur simultaneously in a flock should not be overlooked.

Treatment, Prevention, and Control

There is no specific treatment. Additional knowledge is needed to formulate measures for prophylaxis and control. It is important to know if the flocks are infected or not in view of the possible economic implications for the poultry industry.

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Arbovirus Infections

James S. Guy and Mertyn Malkinson

Introduction

The term *arbovirus*, an abbreviation of *arthropod-borne-virus*, is used to describe a virus that replicates in a hematophagous (bloodsucking) arthropod and is transmitted by bite to a vertebrate host. Taxonomically, the term has been used to group those viruses that share the property of transmission by arthropod vectors. The most recent edition of the *International Catalog of Arboviruses* (60), published in 1985, lists 504 recognized arboviruses; however, an additional 30 arboviruses have been officially recognized since that publication (61). Over 100 arboviruses have been isolated from avian species or ornithophilic arthropod vectors. However, only five arboviruses—eastern equine encephalitis (EEE) virus, western equine encephalitis (WEE) virus, Highlands J (HJ) virus, West Nile (WN) virus and Israel turkey meningoencephalitis (IT) virus have been identified as causes of disease in domestic poultry and farm-reared game birds.

Public Health Significance

Eastern equine encephalitis virus, WEE virus and WN virus are zoonotic agents and potential causes of significant neurological disease in human beings; these infections may progress to paralysis, convulsions, coma and death. The case fatality rate for EEE virus in human beings is 50–75%, and survivors often have permanent neurological sequelae (mental retardation, seizures, impaired motor activity, impaired speech and hearing) (90). Western equine encephalitis virus and WN virus are less severe, with most infections being subclinical. The case-fatality rate in human beings for WEE virus and WN virus is approximately 3–7% and 4–11%, respectively (68, 90). For WN virus infections, it has been estimated that approximately 20% of human infections result in symptomatic illness, and about 1% lead to encephalitis, meningitis, or acute flaccid paralysis (126).

Human infection usually is acquired by mosquito bite; laboratory and clinically acquired infections are rare. However, care should be taken to avoid contact or droplet exposure when handling suspect infected birds or performing necropsies. HJ virus and IT virus are not believed to be pathogenic for human beings.

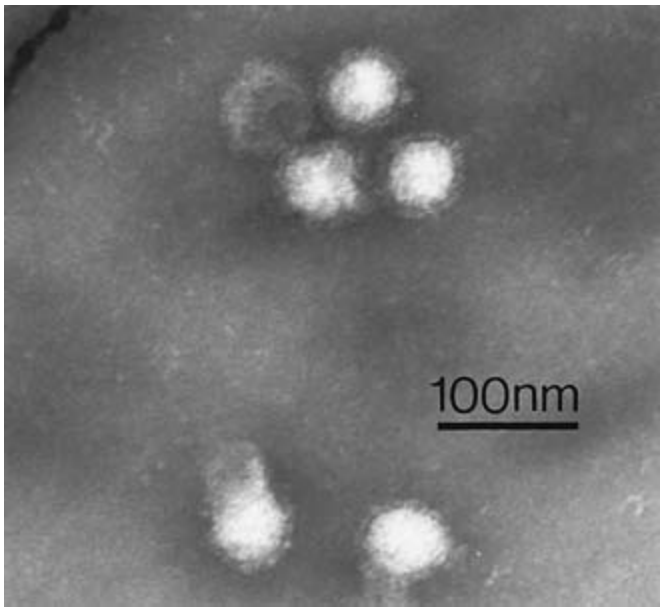
Etiology

Classification

The arboviruses comprise a large, diverse group of viruses, with members in 12 different virus families; however, only the Togaviridae and Flaviviridae contain viruses that cause disease in poultry and game birds. The main characteristics of the Togaviridae and Flaviviridae are presented below.

Togaviridae

Togaviruses are spherical enveloped viruses approximately 70 nm in diameter (Fig. 14.5). The genome consists of a single molecule of positive-sense, single-stranded RNA of 9.7 to 11.8 kilobases (kb), enclosed within a 40-nm diameter icosahedral nucleocapsid (119). Virions are composed of two or three envelope proteins (E1, E2, and sometimes E3) that are usually glycosylated, and a fourth capsid (C) protein. The molecular weights (mw) of alphavirus E1 and E2 structural proteins are 45–58 kilodaltons (kDa); that of E3, when present, is 10 kDa, and C is 30–33 kDa (119). Togaviruses replicate in the cytoplasm, and assembly involves budding of nucleocapsids through host-cell plasma membranes. Some togaviruses exhibit pH-dependent hemagglutinating activity.



14.5. Negative-contrast electron micrograph of eastern equine encephalitis virus. ×150,000.

Togaviridae comprise two genera, *Alphavirus* and *Rubivirus*, but only the *Alphavirus* genus contains arboviruses (119). The alphaviruses formerly were known as the arbovirus A group; the genus includes 29 viruses, the best known being EEE virus, WEE virus, Venezuelan equine encephalitis (VEE) virus, and HJ virus. Based on serologic cross-reactivity (59, 119), alphaviruses have been subdivided into eight antigenic groups named for prototype viruses: EEE virus, WEE virus, VEE virus, Semliki Forest virus, Ndumu virus, Trocara virus, Middleburg virus and Barmah Forest virus. Individual viruses are placed within these antigenic groups based on demonstration of antigenic relatedness to a prototype virus.

Flaviviridae

Flaviviruses formerly were known as the group B arboviruses and until recently were classified in the family Togaviridae. They

now are recognized as a distinct virus family based on differences in virion structure, gene sequences, morphogenesis and replication strategy (110). Flaviviruses resemble togaviruses, with the exception that they are somewhat smaller: approximately 50 nm in diameter. They replicate in the cytoplasm and acquire a lipid envelope by budding into cytoplasmic vesicles. The genome consists of a single molecule of positive-sense, single-stranded RNA of approximately 11 kb. Virions are composed of three structural proteins (64, 110): an envelope (E) glycoprotein with a molecular weight of 51–59 kDa, a core (C) protein of 13–16 kDa, and a membrane-like (prM or M) protein of 7–9 kDa. Flaviviruses exhibit pH-dependent hemagglutinating activity.

The Flaviviridae comprise three genera, *Flavivirus*, *Pestivirus* and *Hepacivirus*, but only the *Flavivirus* genus contains arboviruses (110). The *Flavivirus* genus contains about 70 virus members grouped antigenically into eight antigenic complexes (19, 71). The Japanese encephalitis virus antigenic complex includes WN virus, Japanese encephalitis virus, St. Louis encephalitis virus, and Usutu virus (36, 120). IT virus has been assigned to the Ntaya antigenic complex.

Laboratory Host Systems

Day-old chickens, newborn and baby mice are highly susceptible to arboviruses when inoculated by the intracerebral (IC) route and some are susceptible following inoculation by peripheral routes (88, 101). Intracerebral inoculation of newborn mice, 1 to 4 days of age, is the preferred method for isolation of these viruses. Arboviruses also may be propagated in embryonated chicken eggs and in a variety of vertebrate and arthropod cell cultures. Vero cells, BHK-21 cells, and primary cultures of chicken and duck cells are frequently used for virus propagation. Cytopathic effects are readily produced by arboviruses in vertebrate cell cultures; they are not always produced in arthropod cell cultures.

Arbovirus Diseases of Birds

Five arboviruses have been identified as causes of disease in domestic poultry and farm-reared game birds: eastern equine encephalitis (EEE) virus, western equine encephalitis (WEE) virus, Highlands J (HJ) virus, Israel turkey meningoencephalitis (IT) virus and West Nile (WN) virus.

Eastern Equine Encephalitis

History

Eastern equine encephalitis virus was first isolated in 1933 from the brain of a horse with encephalitis (109). In 1938, the virus was identified by Tyzzer *et al.* (113) as the cause of an epornitic disease of penned pheasants. It was subsequently identified as a cause of disease in pigeons in 1938 (33), chukar partridges (83) and Pekin ducks (28) in 1960, and turkeys in 1961 (103).

Pathogenesis and Epiemiology

Incidence and Distribution

Eastern equine encephalitis is most commonly seen as a disease of horses. Many outbreaks of EEE in farm-raised ring-neck pheasants and chukar partridges have been identified, but it occurs only sporadically in other species of poultry and game birds. The disease occurs primarily in the eastern parts of North America, throughout Central America and the Caribbean, and in eastern parts of South America. In the United States, EEE has been identified in most states east of the Mississippi River, as well as Louisiana and Texas; it occurs most often in Atlantic seaboard states and Gulf Coast states. Reported isolations of EEE virus in Europe and Asia have not been confirmed.

Outbreaks generally occur in late summer and fall as a consequence of increasing numbers of mosquito vectors. Wallis *et al.* (118) demonstrated that increased population densities of mosquitoes coincided with the appearance of outbreaks. Hayes and Hess (44) studied weather patterns associated with EEE outbreaks in Massachusetts and New Jersey and noted that excessive rainfall during the preceding autumn months influenced the occurrence of the disease.

Natural and Experimental Hosts

Outbreaks of EEE in avian species have been reported primarily in pheasants (58, 113); however, outbreaks in pigeons (33), chukar partridges (83, 93), turkeys (32, 103, 115), and ducks (28) also have been reported. Episodes of clinical disease in chickens and quail have not been reported, but both species are highly susceptible to experimental infection (112, 113).

Transmission, Carriers, Vectors

Culiseta melanura, an ornithophilic mosquito, has been determined to be the principal enzootic vector of EEE virus in North America (20, 51). The virus also has been identified in a variety of other mosquitoes including *Aedes sollicitans, Coquilletia perturbans, Culex (Cx.) pancossa, Cx. dunni,* and *Cx. sacchettae,* as well as mites, lice, simuliid flies, and culicoides (24, 116, 117). *C. melanura* is the likely vector responsible for transmission to poultry and game birds; transmission to mammalian species most likely occurs by other mosquitoes such as *Aedes* spp. and *Coquillettia* spp., which feed on birds but also have a propensity to bite mammals (82).

Wild birds, primarily the smaller species of Passeriformes, are the principal vertebrate hosts of EEE virus (66, 82, 122). These birds rarely become ill but serve as maintenance and amplifying hosts for the virus in the transmission cycle. In experimental studies, a variety of wild birds were shown to develop viremia lasting up to 4 days; small passeriform birds were shown to develop viremias with maximal lethal-dose—50% (LD_{50}) titers greater than 10^6/mL (66).

Although EEE virus is transmitted principally by mosquitoes, direct transmission has been shown to occur among pheasants as a result of feather picking and cannibalism (50). In addition, pheasants have been experimentally infected by oral inoculation of the virus (97). Epornitics of EEE virus infection in pheasants are believed to be initiated by mosquito-borne infection of one or more birds in a flock, with subsequent spread within the flock occurring as a result of feather picking and cannibalism.

Transmission of EEE virus by semen also has been demon-

strated (41); virus was shed in the semen of experimentally infected tom turkeys on days 1 to 5 postinfection (PI). Semen collected from infected tom turkeys at 1–2 days PI resulted in transmission to breeder hens after artificial insemination.

Clinical Signs and Pathology

Clinical disease produced by EEE virus in poultry and game birds usually is attributed to central nervous system (CNS) infection with or without involvement of viscera. However, EEE virus also may produce visceral infections with little or no involvement of CNS tissues.

Pheasants

Naturally infected pheasants develop signs of neurologic dysfunction consisting of depression, leg paralysis, torticollis, and tremors (8, 113). Clinical signs occurred in 40–100% of experimentally infected pheasants with mortality of 25–100% (43, 65, 97). Mortality rates up to 80% have been described for naturally occurring outbreaks.

Tyzzer *et al.* (113) and Jungherr *et al.* (58) described the pathology of EEE in pheasants. Gross lesions were not observed; however, histopathologic changes in the CNS consisted of vasculitis, patchy necrosis, neuronal degeneration, and meningeal inflammation.

Turkeys

Outbreaks of EEE in turkeys in Wisconsin were characterized by drowsiness, incoordination, progressive weakness, and paralysis of legs and wings (103). Mortality in affected flocks was low, generally less than 5%. Infected turkeys had neurologic lesions consisting primarily of calcification of blood vessel walls in the cerebral cortex, the cerebellar folia, and the basal part of the medulla. Central nervous system lesions in intracerebrally inoculated birds included lymphocytic perivascular infiltration, neuronal degeneration, and endothelial cell swelling. Calcification of blood vessel walls was not observed in intracerebrally inoculated birds that died before 6 days PI.

Ficken *et al.* (32) serologically identified EEE virus as the cause of high mortality in young (1- to 4-week-old) turkeys. Subsequent experimental studies demonstrated susceptibility of young turkeys to experimental infection (38). Two-week-old turkeys experimentally infected with EEE virus exhibited depression, somnolence, and high mortality. Viremia was detected in infected turkeys on days 1 and 2 PI, with peak viremia of $10^{5.5}$ plaque-forming units per ml (PFU/ml) detected on day 1 PI. Pathologic changes consisted of multifocal necrosis in the heart (Fig. 14.6A), kidney, and pancreas, and lymphoid necrosis and depletion in the thymus (Fig. 14.6B), spleen and bursa of Fabricius (Fig. 14.6C). No lesions were detected in brains.

Acute drops in egg production in turkey breeder hens due to EEE virus infection were reported by Wages *et al.* in 1993 (115). Decreased egg production in affected flocks was characterized by sudden onset of production of white, thin-shelled and shell-less eggs. No increase in mortality was observed, and acute ovarian regression was the only gross lesion observed. Experimental infection of turkey hens with EEE virus reproduced the disease observed in naturally affected flocks (40). Eastern equine encephalitis virus-infected turkey hens exhibited mild depression and inappetence, but only on day 1 PI. A precipitous decline in egg production began on day 2 PI, and production remained depressed for 15 days; no mortality was observed. Viremia of short duration (1–2 days), peaking at $10^{5.8}$ PFU/ml on day 1 PI, was detected in EEE-virus-infected hens.

Chukar Partridges

Chukar partridges infected with EEE virus exhibited clinical signs of depression, somnolence, and high mortality (30–80%) (93). Pale, focal areas were present in hearts of affected birds, and spleens were mottled and enlarged. Microscopic lesions consisted of gliosis, satellitosis, and perivascular lymphocytic infiltration in brains, and myocardial necrosis with lymphocytic infiltration.

Ducks

White Pekin ducklings infected with EEE virus developed a paralytic disease characterized by sudden onset, posterior paresis, and paralysis (28). Mortality rates in EEE virus-affected flocks were 2–60%. Histopathologic lesions consisted of edema of spinal cord white matter, lymphocytic meningitis, and microgliosis.

Chickens

Newly hatched chickens are highly susceptible to EEE virus and succumb rapidly to the infection, often without showing signs of CNS involvement. Byrne and Robbins (16) demonstrated that susceptibility of chickens to lethal EEE virus infection declined rapidly with age; in their study, chickens became refractory to lethal infection by 14 days of age. In contrast to their findings, susceptibility to lethal infection was demonstrated by Tyzzer and Sellards (112) in 3- to 13-day-old chickens and by Guy *et al.* (39) in 14-day-old chickens. The different findings from these studies have not been explained, but differences in age-dependent resistance may be due to differences in host genetics and/or differences in virulence of the EEE viruses used in these studies.

Experimental infection of young chickens, 1–14 days of age, caused depression, somnolence, and high mortality; paralysis was infrequently observed (39, 112). The principal lesion, and the presumed cause of death, was myocarditis. Microscopic heart lesions consisted of multifocal necrosis with fragmentation of myocardial fibers, and infiltration with lymphocytes, plasma cells, and macrophages (Fig. 14.6E). Central nervous system lesions in infected chickens were inconsistently observed (39, 112). In brains, microscopic lesions consisted of occasional small foci of necrosis and mild perivascular cuffing (Fig. 14.6D). Multifocal necrosis of the liver (Fig. 14.6F) and lymphoid depletion and necrosis in the thymus, spleen, and bursa of Fabricius also were observed in EEE virus-infected chickens (39). Ascites and right ventricular dilatation of the heart were observed in chickens that survived the acute effects of EEE virus infection; these effects likely occur due to myocardial damage (39).

Diagnosis

Isolation and Identification of Causative Agent

Diagnosis of eastern equine encephalitis may be accomplished by isolation and identification of the virus, detection of viral antigens using antigen-capture enzyme-linked immunosorbent assays (ELISAs) (48, 49, 98, 99), or immunohistochemistry (123), detection of viral RNA using reverse transcriptase, polymerase chain reaction (RT-PCR) procedures (114), and serologic testing (101). The virus can be isolated by inoculation of blood or tissue homogenates (brain, spleen, liver, heart) into newborn mice by the intracerebral route, day-old chickens by subcutaneous or intramuscular routes, and 5- to 7-day-old embryonated chicken eggs by the yolk sac route (88, 101). In addition, a variety of cell cultures may be utilized for isolation of the virus; Vero, BHK-21, and chicken or duck embryo cells are highly susceptible. Newborn mice and 1-day-old chickens generally die of encephalitis in 2–5 days. Chicken embryos generally die within 18–72 hr and have a hemorrhagic appearance. Cell cultures develop cytopathic effects (CPE) within 24–48 hr, and plaques develop under agar within 36–48 hr. Identification of EEE virus in inoculated animals, embryonated eggs, or cell cultures generally is accomplished by virus-neutralization (VN) tests or complement fixation (CF) tests.

Antigen-capture ELISA procedures for detection of EEE virus antigens have been described (14, 48, 49, 98, 99). These procedures have been shown to be highly sensitive, detecting EEE virus in experimentally infected birds as early as 12 hr PI and in pools of insects in which only 1% of the insects were infected (14, 48, 49, 98, 99). Commercially available antigen-capture ELISA tests recently have become available for detecting EEE virus in mosquitoes; these also are available for detection of WEE virus and WN virus (85).

An immunohistochemical procedure (123) and a RT-PCR (114) were described for detection of EEE virus antigens and viral RNA, respectively, in tissues of infected birds. These procedures were shown to be rapid, sensitive and specific methods for detection of EEE virus in tissues. Additionally, these diagnostic procedures, along with ELISA, minimize the human health risks inherent with virus isolation and identification procedures.

Serology

Serological diagnosis of EEE virus may be accomplished using VN, hemagglutination-inhibition (HI), ELISA, and CF. Of these, VN and HI tests are most commonly utilized. The HI test is rapid and relatively simple; it requires either goose or 1-day-old chicken erythrocytes, and antigen prepared from infected suckling mouse brains by the sucrose-acetone extraction method (21, 101). Avian serum contains nonspecific inhibitors of hemagglutination and these must be removed by kaolin adsorption before use in HI tests. A presumptive serologic diagnosis may be obtained by detection of EEE virus antibodies in serum collected from recovered birds. A definitive diagnosis is achieved by demonstrating a rising antibody titer in serum samples collected soon after onset of clinical signs and 1–2 wk later.

Guy *et al.* (40) demonstrated the value of serology for diagnosis of EEE virus-induced episodes of decreased egg production in turkey breeder hens. Serology was shown to be particularly important because the virus was found to be present in most tissues of experimentally infected turkey breeder hens for only a very brief period (on days 1–2 PI) following experimental inoculation, yet marked drops in egg production became apparent only after day 2 PI.

Differential Diagnosis

Eastern equine encephalitis must be distinguished from other causes of neurologic disease in poultry and game birds such as HJ virus, Newcastle disease virus, avian encephalomyelitis virus, botulism, and listeriosis. In cases of egg-production drops in turkeys, EEE virus, WEE virus, HJ virus, Newcastle disease virus, avian influenza virus, avian encephalomyelitis virus, paramyxovirus type 3, turkey coronavirus, and turkey rhinotracheitis virus must be considered. These diseases generally are distinguished based on isolation and identification of the causative agent or by serologic analyses.

Intervention Strategies

Eastern equine encephalitis is best prevented and controlled by measures aimed at reducing vector populations. Such measures include reduction of vector habitats by modifications of the environment or by chemical spraying. If feasible, farms that raise susceptible avian species should be located away from swamps and other areas that provide habitat for vectors.

Formalin-inactivated EEE vaccines, prepared for use in horses, have been used to protect pheasants against EEE epornitics (105), although their efficacy has been questioned (29).

Western Equine Encephalitis

Western equine encephalitis virus has many characteristics in common with EEE virus. The virus is rarely associated with disease in avian species; however, a few cases have been reported (23, 31, 93, 124). In 1957, WEE virus was identified by Woodring (124) as the cause of encephalitis and high mortality in turkeys in Wisconsin based on serologic studies; affected turkeys exhibited somnolence, tremors, and leg paralysis. Faddoul and Fellows (31) reported the isolation of WEE virus from the brain of a pheasant in Massachusetts, and Ranck *et al.* (93) identified the virus as the cause of high mortality in chukar partridges in Florida. However, the association of WEE virus with disease in these species, particularly pheasants (31) and chukar partridges (93), is tenuous. It is now generally accepted that WEE virus does not occur in the eastern United States, and that all WEE-related alphaviruses isolated in the eastern United States are actually strains of HJ virus (see below) (17, 111).

Recently, WEE was associated as a cause of decreased egg production in turkey breeder hens in California (22). Decreased egg production in affected flocks was characterized by sudden onset with production of small, white-shelled and shell-less eggs. No increase in mortality and no clinical signs were observed in affected flocks. A WEE virus isolated from affected breeder hens

was evaluated for pathogenicity in 2-week-old turkeys (23). The isolate failed to produce clinically apparent disease in inoculated turkeys, but infection resulted in mild to moderate lymphoid necrosis in the bursa of Fabricius and thymus.

Western equine encephalitis is identified mainly in western parts of the United States and Canada, in Central America, and in South America. It is transmitted principally by *Culiseta tarsalis,* a mosquito vector that is relatively common in the United States west of the Mississippi River (20). Laboratory diagnosis of WEE is accomplished using the same procedures that are used for EEE.

Highlands J Virus Infection

Highlands J virus initially was isolated in 1960 from blue jays in Florida (47). Since that time, the virus has been identified as a cause of disease in chukar partridges (30, 93) and turkeys (32, 38, 40, 115).

Ranck *et al.* (93) reported that WEE virus was the cause of mortality in chukar partridges in Florida in 1964; however, this virus most likely was HJ virus. Antigenically, HJ virus is closely related to WEE virus and for many years was considered to be a variant of that virus (45, 47, 62). However, serologic and oligonucleotide mapping studies clearly differentiate these viruses and have identified HJ virus as a distinct virus in the WEE antigenic group of alphaviruses (17, 18, 59, 111). All viruses belonging to the WEE antigenic group that have been isolated in the eastern United States have been determined to be HJ virus (17).

Ranck *et al.* (93) experimentally reproduced the disease by subcutaneous inoculation of young chukars. Experimentally infected chukars exhibited somnolence, ruffled feathers, and recumbency prior to death; lesions primarily consisted of encephalitis and myocardial necrosis. Eleazer and Hill (30) described a more recent outbreak of HJ virus infection in chukar partridges in South Carolina. Chukars exhibited similar clinical signs and high mortality (35%); myocarditis was consistently observed in affected birds, but lesions in the brain were uncommon.

Wages *et al.* (115) found HJ virus to be the cause of acute drops in egg production in turkey breeder hens. In addition, these viruses were serologically associated with mortality in young turkeys (32). Experimental infection of turkeys with HJ virus produced precipitous egg production drops in turkey hens (40), but was only mildly pathogenic for young turkeys (38). The clinical and pathologic characteristics of HJ virus infection in turkeys closely resemble those of EEE virus infection (see above).

Laboratory diagnosis of HJ virus infection is accomplished using the same procedures used for EEE virus and WEE virus. These include virus isolation, serology, antigen-capture ELISA, and RT-PCR procedures (32, 40, 121). Highlands J virus is readily distinguished from WEE virus isolates by a variety of serologic procedures using polyclonal and monoclonal antibodies (63).

Israel Turkey Meningoencephalitis

History

Israel turkey meningoencephalitis (IT) was first described in Israel by Komarov and Kalmar in 1960 (70). In 1961, Porterfield (91) identified the etiologic agent as a new virus belonging to the Flaviviridae. Based on its serological properties IT virus was assigned to the Ntaya serogroup (19). The disease was identified in South Africa in 1978 (7).

Incidence and Distribution

Israel turkey meningoencephalitis has been reported only in Israel and South Africa. Outbreaks of the disease occur seasonally in all regions of Israel, corresponding with the activity of arthropod vectors; outbreaks generally begin in late summer, peak in October, and disappear in early winter (52).

Pathogenesis and Epidemiology

Natural and Experimental Hosts

Israel turkey meningoencephalitis has been reported only in turkeys. Field cases of IT are rarely observed in turkeys less than 10 wk of age, but younger birds are equally susceptible (95). Experimental infection of turkeys less than 10 wk of age results in disease with an incubation period of 5 to 8 days (52). A viremia is detectable within 24 hr PI in experimentally infected turkeys and persists for 5–8 days (55).

Newly hatched poults (53), Japanese quail (*Coturnix coturnix japonica*) (56) and suckling mice (53) are highly susceptible to IT virus inoculated by the intracerebral and intramuscular routes. Chickens, ducks, geese, and pigeons are refractory to infection (70).

Transmission, Carriers, Vectors

The seasonal incidence and sporadic occurrence in flocks on the same farms strongly suggest that IT virus is transmitted by insect vectors. The virus has been isolated from unsorted pools of mosquitoes (*Aedes* spp. and *Culex pipiens*) and culicoides trapped near affected turkey flocks (12). Experimentally, IT virus has been shown to infect *Aedes aegypti* and *Culex molestus* mosquitoes (87). Field observations and experimental studies indicate that virus transmission does not occur by direct contact between infected and uninfected birds (55, 57).

Clinical Signs and Pathology

In field outbreaks, IT occurs with greatest incidence in turkeys 10 to 12 weeks of age. Affected turkeys exhibit neurologic dysfunction characterized by progressive paresis and paralysis, with variable mortality. Morbidity and mortality rates generally average 15–30% but may be as high as 80% (52). Affected birds initially exhibit an uncoordinated gait and walk with one or both wings drooping. As the disease progresses, birds become reluctant or unable to walk, and rest on their breasts with legs extended forward and wings spread laterally. Turkey breeder hens exhibit a severe drop in egg production, but egg quality, fertility, and hatchability are unaffected. Egg production returns to normal levels after recovery from infection.

Gross lesions include splenomegaly or atrophy of the spleen, catarrhal enteritis, and myocarditis (6, 54, 70). Ovarian regression, ruptured ovarian follicles, and peritonitis are observed in affected breeder hens (6). The principal microscopic lesions are

nonpurulent meningoencephalitis characterized by submeningeal and perivascular lymphocytic infiltration, and focal myocardial necrosis (54, 70).

Diagnosis

Brain, spleen, liver, serum, and ovary are the preferred materials for virus isolation (53, 55). Homogenates of tissue or undiluted serum are inoculated into 6- to 8-day-old embryonated chicken eggs by the yolk sac route, or onto monolayers of chicken embryo fibroblasts (CEF). One or more passages in embryonated chicken eggs may be required before embryo mortality is observed; embryos die 3 to 6 days PI and show a distinct cherry-red discoloration. Suckling mice inoculated by the intracerebral or intramuscular routes also may be used for virus isolation (53). A specific RT-PCR assay for IT virus recently has been described (25).

A readily recognizable CPE is produced in infected CEF cells by 3 days PI (54, 55); however, CEF cells are less sensitive than embryonated chicken eggs or suckling mice for isolation of IT virus. Identification of isolates usually is accomplished by VN tests.

Serological diagnosis can be accomplished using HI or VN tests in CEF or BHK-21 cells (7, 54, 55, 89). The HI test requires either goose or day-old chicken erythrocytes and antigen prepared from infected suckling mouse brains by the sucrose-acetone extraction method (53).

Differential Diagnosis

Israel turkey meningoencephalitis must be differentiated from other causes of neurological disease in turkeys, particularly Newcastle disease virus, highly pathogenic avian influenza virus, EEE virus, and HJ virus. The known geographic distribution of these viruses and the greater severity of paralysis that is observed with IT as compared with EEE and HJ are helpful in distinguishing these agents. Nervous signs may be observed with Newcastle disease, but paralysis generally does not occur. Nervous signs also may be caused by *Riemerella anatipestifer* infection and ionophore toxicity.

Control

Israel turkey meningoencephalitis is controlled by vaccination. Live attenuated vaccines have been prepared by serial passage of IT virus in embryonated chicken eggs (54), Japanese quail kidney cells (57), and BHK-21 cells (7). The Japanese quail kidney cell-attenuated virus has been shown to be highly efficacious and is commercially available. Reduction of insect vector populations in the vicinity of turkey farms also may be useful in controlling the disease. IT virus does not cause disease in humans.

West Nile Virus

History

West Nile (WN) virus was first isolated from the blood of a febrile Ugandan woman in 1937 (102). The virus was first described as the cause of a WN fever epidemic in humans in Israel in 1951 (9, 35), and in a later outbreak, severe meningoencephalitis was seen in elderly patients. The role of mosquitoes in viral transmission was clearly delineated in a series of field studies in Egypt in the 1950s (108); the involvement of various wild birds as reservoirs of the virus also was described during this period (125). Cases of WN fever in horses were reported in Egypt and France several years later. West Nile virus was first identified as a significant cause of disease in domestic avian species in 1997, when the virus was identified as a cause of neurological disease in young geese (75). In August 1999, the disease was detected for the first time in the Western Hemisphere in wild birds, zoo birds, horses and human beings in the United States (104).

Pathogenesis and Epidemiology

Incidence and Distribution

WN virus is now considered to be endemic in many countries of Africa, Asia, southern Europe, North America and Central America (46, 104). Epidemics appear in the human population at infrequent intervals in some of these countries and there is evidence for viral transmission bidirectionally between Africa and Europe by migrating birds (76). Epidemics occurred for the first time in Romania in 1996 and Russia in 1999, in which several hundred people were affected and the case fatality rates reached 10% or more. In 1998 and 2000, outbreaks affecting horses were seen in Italy and southern France respectively. In Israel, outbreaks affecting geese reappeared in 1998, 1999, and 2000; an epidemic affecting 500 people with 29 deaths occurred in 2000.

West Nile viruses isolated from different parts of the world were shown to segregate into two distinct lineages based on complete genomic sequences (72) and sequence analyses of the E protein gene (10). Lineage I contained WN viruses isolated in Europe and Africa; lineage II contained viruses isolated in Africa, Madagascar, and most recently in Central Europe (4).

Natural and Experimental Hosts

Outbreaks of WN in poultry have been reported primarily in geese (3, 5, 34, 75, 79). Episodes of clinical disease in chickens and turkeys have not been reported, but both species are susceptible to experimental infection (100, 106). Experimental infection of young Muscovy ducks resulted in mortality.

A wide variety of feral and captive birds are known to be susceptible to WN virus infection (68). In a study examining the role of various feral birds as reservoirs of WN virus in the transmission cycle, 25 species were infected experimentally with a New York 1999 strain of the virus (69). Based on the levels of viremia, the five most competent species were passerines: blue jay (*Cyanocitta cristata*), common grackle (*Quiscalus quiscula*), house finch (*Carpodacus mexicanus*), American crow (*Corvus brachyrhynchos*), and house sparrow (*Passer domesticus*).

Transmission, Carriers, Vectors

The principal route of transmission is by the bite of a *Culex* mosquito. In the USA during 1999 and 2000, most of the viral isolates were made from *Cx. pipiens* and *Cx. restans* (37). In Africa and the Middle East, the usual vector is *Cx. univittatus* and in

Europe, *Cx. pipiens* and *Cx. modestus*. WNV has been isolated from at least 10 tick species belonging to *Amblyomma*, *Dermacentor*, *Hyalomma*, *Rhipicephalus*, *Argas*, and *Ornothodorus* genera (84). West Nile virus was found in hibernating *Cx. pipiens* found in New York City and in male *Cx. univittatus* in Kenya indicating that transovarian transmission occurs in nature (80).

Most outbreaks begin in mid-July and end in October when cold nights reduce mosquito vector activity, notably *Culex* species. Outbreaks of WN in goose flocks have also been reported in Canada (3), Hungary (34), and the USA (79).

Until recently, wild birds were considered to be only sporadic victims of WN virus infection and evidence for their role as carriers was gained primarily from serological surveys. The isolation of virus during the 2000 epizootic from white storks, gulls, feral pigeons and a wide range of American birds including crow, jay, dove and hawk indicates that wild species actively become infected and also transmit the virus over considerable distances as they migrate. Viral titers in crow blood, for example, exceeded 10^{10} plaque-forming units/ml (2). Based on experimental data, direct transmission between crows is suspected to occur in communal roosts resulting in widespread dispersion of the virus in nature (78).



14.7. Six-week-old geese infected with West Nile virus. The bird on the left is unable to stand, and the bird on the right has spread its wings in attempt to retain its balance (Weisman).

Incubation Period

Mortality was observed in geese experimentally infected by the intracerebral route beginning on day 5 PI, and on day 8 PI after experimental infection by the intramuscular route. In naturally infected flocks, mortality rates of 10% to 60% have been reported (4, 34); these high rates may be due in part to horizontal spread of the virus. In another experiment, an in-contact goose was found to be viremic on day 10 PI having been infected by cage mates inoculated with a crow isolate of WN virus (107).

Clinical Signs and Pathology

Geese

West Nile virus-infected geese show various degrees of neurological involvement ranging from recumbency to leg and wing paralysis (Fig. 14.17) (34, 107). Affected birds are either reluctant or unable to move when disturbed. Signs of incoordination are pronounced; some birds may fall while attempting to stand. Torticollis and opisthotonus may be observed.

In 3–4 wk old geese experimentally infected by the subcutaneous or intramuscular route, viremia was detected in some birds as early as day 1 PI. Peak viremias of 10^4–10^6 tissue culture doses/ml occurred on days 2–4 PI; viral titers declined or disappeared coincident with the appearance of neutralizing antibodies. Some geese had detectable VN antibodies by day 4 PI. In subcutaneously infected 2-wk-old goslings, WN virus was recovered from the plasma of one in-contact bird on day 10 PI and VN antibody was detected on day 14 and on day 21 PI in this gosling (107). Viral excretion from inoculated geese in this experiment was determined to be from the oropharynx and not from feces. The high viremic levels in infected geese are sufficient to transmit virus to engorging mosquitoes; geese thereby act as reservoirs for further circulation.

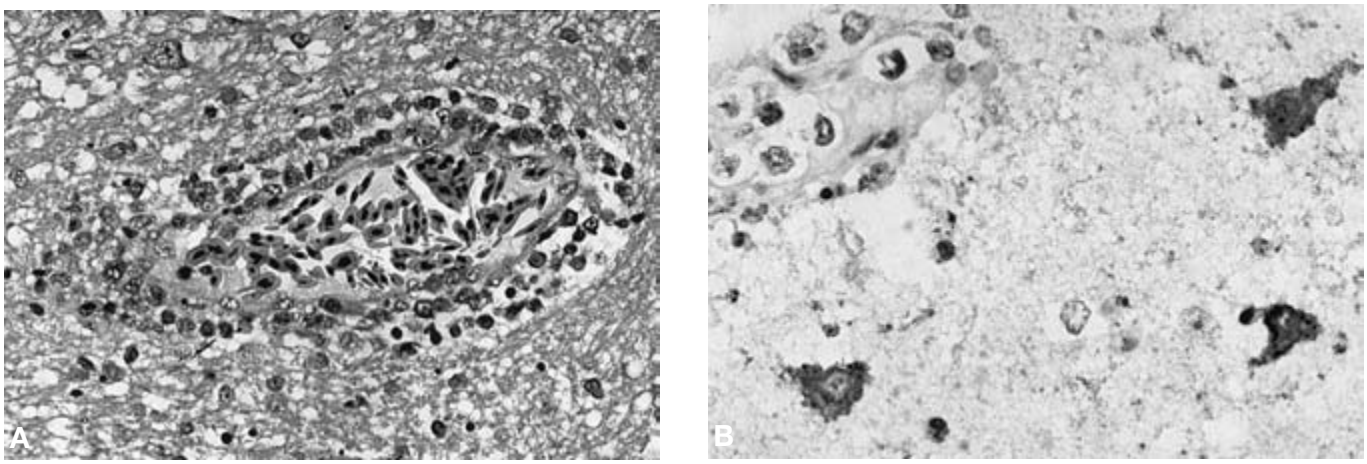
During a recent epizootic, losses due to WN virus infection in young goose flocks were estimated to be far greater than expected if mosquito-borne transmission was the principal route of infection. Contact transmission was investigated experimentally as an alternative explanation (5). A group of 3-week-old geese were inoculated subcutaneously and placed in an insect-proof room with 20 geese of the same age. All geese in the inoculated group produced antibodies, eight became viremic and five died between 7 and 10 days after infection. Virus was shed from the cloaca and oral cavity by three geese. Two of the in-contact birds died on days 10 and 17 after infection, and WNV was recovered from another three birds. These findings strongly suggest that horizontal transmission of WN virus can occur in commercial flocks and may be aggravated if cannibalism and feather-picking of sick geese occur.

Pathological changes in WN virus-infected geese include pallor of the myocardium and occasionally the kidneys, splenomegaly and hepatomegaly; engorgement of meningeal blood may be observed. Microscopic lesions were found mainly in the brain and consisted of lymphocytic perivascular infiltration and neuronal degeneration (Fig. 14.8). Small necrotic foci were present in the heart muscle but lymphocytic infiltration was minimal.

Chickens

Day-old chickens develop neurological signs including tremors and paralysis following inoculation by a variety of routes. These signs appeared between 5–10 days PI (94). Chickens aged 1–11 days developed viremia of 10^4–$10^{6.3}$ mouse infectious doses/ml following infection by mosquito bite and were capable in turn of infecting mosquitoes (108). In endemic areas chickens became naturally infected and a serological prevalence rate approaching 20% has been recorded. Sentinel chickens also play an important role in serological surveillance programs and have been used extensively in the USA (67) and England (15).

Experimentally infected 7-week-old chickens developed viremia of 10^5 tissue culture doses/ml on day 5 PI that persisted



14.8. Microscopic lesions in the brain of West Nile virus-infected goose. A. Perivascular cuffing by mononuclear cells (H&E stain) (Perl). B. Immunohistochemistry. Three intensely stained neurons with viral antigen in the cytoplasm; the nuclei remain unstained. Stained granules are dispersed in the neurophil. Counter-staining with hematoxylin (Perl). Monoclonal antibody supplied by Dr. Vincent Deubel, Pasteur Institute, Paris.

until day 7 PI. Some chickens shed virus in their feces on days 4 and 5 PI (100). Nevertheless, neither clinical signs nor mortality were seen in birds infected subcutaneously with an American crow isolate (100). This level of viremia is enough to infect engorging mosquitoes. Birds sacrificed on days 5 and 10 PI showed myocardial necrosis, nephritis and peritonitis, while at termination of the experiment on day 21 PI non-suppurative encephalitis was found. No viral transmission to in-contact chickens was detected; they remained antibody and viremia negative for 21 days. In the light of these observations, young chickens should be viewed as potential amplifying or reservoir hosts.

Turkeys

No morbidity or mortality has been reported in commercial turkey flocks. When 3-wk-old poults were experimentally infected subcutaneously with an American crow isolate none became clinically affected, however, most of them became viremic for up to 10 days PI (106). Virus was re-isolated from feces on days 4–7 PI but in-contact poults were not infected.

Immunity

Geese rapidly develop high titers of circulating antibodies to WN virus; however, these are not reliable indicators of protection. Cell mediated immunity has not been studied in geese; however, geese vaccinated with a live, attenuated IT vaccine were resistant to intracerebral challenge with a field isolate of WN virus, but failed to develop detectable VN antibodies (77). In a mouse model, B cells and antibody were shown to play critical roles in defense against disseminated infection (27).

Maternal antibodies have been detected in sera collected from 1–2 wk old goslings hatched from commercial geese flocks, but these did not interfere with an active response to inactivated WN virus vaccine. Based on field observations, susceptibility of geese to natural infection appears to decline with increasing age; geese older than 12 weeks of age appear to be resistant to disease.

Diagnosis

Isolation and Identification of WNV

Tissues of choice for isolating WN virus from birds are the brain, spleen and kidneys. Tissue homogenates are inoculated into newborn mice by the intracerebral route, into embryonated eggs by the yolk sac route, or onto monolayers of Vero cell cultures or mosquito cell cultures. Mice develop ataxia within 4–7 days; chick embryos die within 2–6 days PI and have an injected appearance. Cell cultures develop a cytopathic effect within 48–72 hrs. Virus may be identified in cell cultures by indirect immunofluorescence; monoclonal antibodies may be used for this procedure and are available commercially, and from reference laboratories.

Reverse transcriptase-polymerase chain reaction procedures recently have been described (10, 73). These procedures allow rapid detection of WN virus in avian tissues, cell cultures, and field-collected mosquitoes. Immunohistochemistry and *in situ* hybridization have been described for detecting WN virus antigens and viral RNA, respectively, in tissues of infected birds (104). These diagnostic procedures minimize the human health risks inherent with virus isolation and identification procedures.

Serology

Serological diagnosis can be accomplished using HI or ELISA tests. A group-specific flavivirus antigen, acetone-extracted from infected mouse brain, forms the basis of the HI test (see EEE section). Several forms of ELISA have been developed for flaviviruses; one variation is to employ a cross-reactive flavivirus monoclonal antibody to block avian sera (42). An indirect ELISA may be utilized in which flavivirus antigen of cell culture-origin is used to coat the plate. In addition, a blocking ELISA has been developed for working with sera from multiple avian species (11). Flavivirus antibodies were detected by ELISA in chicken sera by day 6–10 PI (13).

Differential Diagnosis

Nervous signs in young geese may be caused by a variety of bacteria including *Riemerella anatipestifer*, *Streptococcus gallolyticus*, *Erysipelothrix spp*., *Listeria spp*. and *Salmonella spp*. Neurotropic viruses include Newcastle disease, which is rare in geese, and highly pathogenic avian influenza virus. Nervous signs also may be caused by *Aspergillus spp*. and ionophore intoxication.

Intervention Strategies

Management Procedures

Mosquito control is a mandatory component of any WN control program. Unfortunately this is very difficult to implement because of the distances that mosquitoes can fly, or can be carried by prevailing winds. Nevertheless, standing water and similar insect breeding sites in the vicinity of densely populated avian farms should be treated with larvicides. Poultry houses should be constructed to be insect-free. Because WN is a significant public health threat, much can be achieved by cooperation with public health agencies.

Vaccination

Control of WN virus infections in geese is primarily confined to vaccinating young flocks at risk, especially those raised during July through November when *Culex spp*. are most numerous. Because of age susceptibility, goslings should be immunized as young as possible and preferably at 3 weeks of age. WN vaccines are now commercially available, and several types recently have been developed for use in birds and horses (81, 86, 96).

Field trials have been performed extensively with a formaldehyde-inactivated mouse brain-derived product (77, 96). The production protocol is based on that described for Japanese encephalitis virus vaccine (1). Over 75% of geese vaccinated with a single dose of vaccine at 3 weeks of age were protected and 94% protection was achieved with two doses spaced two weeks apart. The duration of immunity was estimated to be approximately 12 weeks. Inactivated vaccines prepared from chick embryos or Vero cells are less protective because of their low antigenic mass.

WN virus has been attenuated by serial passage in mosquito cell cultures (74). A single dose of mosquito cell-passaged virus induced immunity to intracerebral challenge in young geese. Mosquito feeding experiments and back passage/reversion to virulence studies have not been completed.

The use of IT virus vaccine has been investigated in commercial flocks (77). A single injection given at 3 weeks of age produced protection in geese challenged two weeks later. This is an example of cross-protection that is known to exist within the flavivirus family (92). However, some birds vaccinated with IT virus vaccine developed a post-vaccination paralytic reaction causing losses of up to 10% in some flocks.

A DNA vaccine based on a recombinant plasmid that expresses the preM and E proteins has recently been developed (26). In initial experiments mice and horses were protected against infection and lethal challenge.

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Turkey Viral Hepatitis

James S. Guy

Introduction

Turkey viral hepatitis (TVH) is a highly contagious, generally subclinical disease of turkeys. It is characterized by multifocal hepatic necrosis with or without accompanying pancreatic necrosis.

The economic significance of TVH is not known. There is no evidence to suggest that TVH virus is transmissible to human beings or other mammalian species.

History

Turkey viral hepatitis initially was described in 1959. It was described simultaneously by Mongeau *et al.* (6) in Canada, and Snoeyenbos *et al.* (9) in the United States.

Etiology

The etiologic agent of TVH has not been characterized. It was suggested in 1959 by both Mongeau *et al.* (6) and Snoeyenbos *et al.* (9) to be a virus based on filtration experiments. The agent was demonstrated by Mongeau *et al.* (6) and by Tzianabos and Snoeyenbos (11) to pass a 100 nm filter.

Based on morphology, site of replication and antigenic analyses, TVH virus likely is a picornavirus (2, 3, 5, 12). In 1982 MacDonald *et al.* (3) identified aggregates of 24 nm, picornavirus-like particles in the cytoplasm of degenerating hepatocytes in livers from turkeys with hepatitis and pancreatitis. In 1991 Klein *et al.* (2) isolated a picornavirus-like virus, 26–28 nm in diameter, with icosahedral morphology from liver and pancreas tissues collected from TVH-affected turkeys. Turkey viral hepatitis was experimentally reproduced by inoculation of young turkeys with this virus. Antigenic analyses based on agar-gel precipitin tests indicated a one-way antigenic relationship between TVH virus and duck hepatitis virus, a picornavirus (12). Antisera produced in rabbits against TVH virus produced confluent precipitin bands with TVH virus and duck hepatitis virus; rabbit antiserum prepared against duck hepatitis virus did not react with TVH virus. While these studies suggest a picornavirus etiology for TVH, additional studies are needed to definitively classify the virus, particularly biochemical and nucleotide sequence analyses of the viral nucleic acid.

Susceptibility to Chemical and Physical Agents

The virus is resistant to ether, chloroform, phenol, and creoline, but not formalin. In yolk it survives 6 hr at 60°C, 14 hr at 56°C, and 4 wk at 37°C. It survived for 1 hr at pH 2 but not at pH 12 (11).

Laboratory Host Systems

Turkey viral hepatitis virus can be propagated and assayed in embryonated chicken eggs, embryonated turkey eggs, and turkey poults. The virus has not been propagated in cell culture (12).

Propagation of the virus may be accomplished by yolk sac inoculation of 5- to 7-day-old embryonated chicken eggs (6, 8, 9). Attempts to propagate TVH virus in embryonated chicken eggs using older embryos or different routes of inoculation generally have been unsuccessful. Virus was demonstrated in inoculated embryonated chicken eggs at 66 hrs postinoculation and peak virus titers of approximately $10^{3.5}$ EID_{50}/ml were detected at 90 hrs postinoculation (10). The virus also may be propagated by yolk sac inoculation of embryonated turkey eggs up to 10 days of incubation; however, embryonated chicken eggs have been shown to be a superior host system, possibly due to the presence of maternal antibody in turkey eggs (4).

Turkey poults are susceptible to infection by intraperitoneal, intravenous and intramuscular routes of exposure. Clinical signs seldom develop in experimentally infected poults, but infection may be demonstrated 5–10 days PI by necropsy and detection of characteristic lesions (7).

Pathobiology and Epidemiology

Incidence and Distribution

Turkey viral hepatitis has been described in Canada, the United States, Italy and Great Britian (3, 4, 6, 9). The disease is believed to be widely distributed in North America, but the true incidence and distribution is not known owing to the frequent subclinical nature of the disease, and the absence of serologic diagnostic tests.

Natural and Experimental Hosts

Turkey viral hepatitis has been recognized only in turkeys. Chickens, pheasants, ducks, quails, mice and rabbits have been shown to be refractory to infection (12).

Transmission, Carriers, Vectors

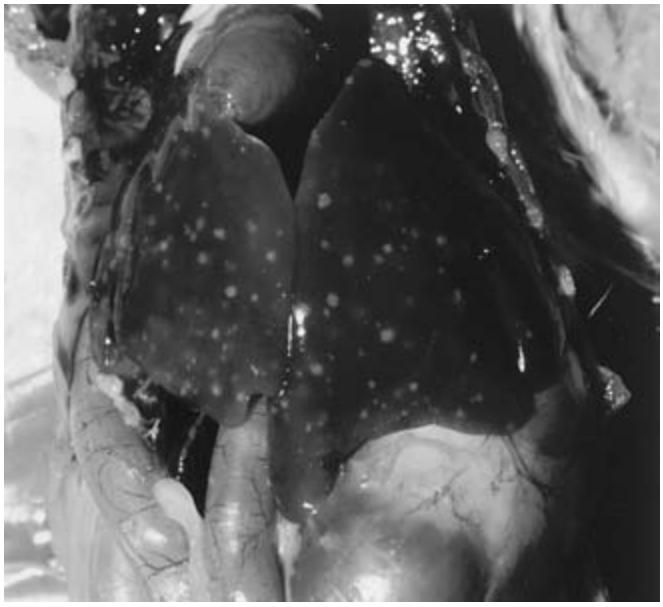
Transmission of TVH virus occurs readily by both direct and indirect contact. Feces from infected turkeys is believed to be the principal source for virus transmission; the virus could be consistently isolated from liver and feces of experimentally infected birds during the first 28 days PI, and less frequently from bile, blood and kidney during this period. The virus could not be detected in tissues and feces after 28 days PI (10, 12). Vertical transmission via the egg has been suggested by field observations and by the isolation of virus from an ovarian follicle of an experimentally infected hen (8).

Incubation Period

The incubation period in poults, as determined by the appearance of lesions, varied between 2–7 days in both intraperitoneally inoculated and in-contact poults (8, 10).

Clinical Signs

Turkey viral hepatitis is usually a subclinical infection of turkeys (3, 7). It is believed that the disease becomes apparent as a result



14.9. Multiple, pale tan to gray foci in the liver of a poult with turkey viral hepatitis. Lesions vary from 1 to several mm. They are randomly scattered throughout the liver, and roughly circular, oval, or elliptical. Lesions often have an irregular, "frayed" border, and some have a darker, slightly depressed central area. (Barnes)



14.10. Poult with turkey viral hepatitis showing prominent pancreatic foci. (Barnes)

of undefined factors such as concurrent infection and/or environmental stresses. Clinical signs in TVH-affected birds are not well defined. Variable degrees of depression may be observed in affected flocks, but more commonly field cases are characterized by sudden death of apparently normal birds. Turkey viral hepatitis virus has been suggested as a cause of decreased egg production, decreased fertility, and decreased hatchability in turkey breeder hen flocks but an etiologic role for TVH virus has not been conclusively determined (7).

Morbidity and Mortality

Morbidity and mortality vary considerably among affected flocks. Usually morbidity and mortality are very low with mortality occurring during a 7- to 10-day period (7). However, morbidity rates of up to 100% have occurred in some flocks, and a 25% mortality was reported in one flock (7). It is believed that severity of morbidity and mortality are influenced by other factors such as concurrent infection. Mortality in turkeys over 6 weeks of age has not been reported.

Pathology

Gross

Gross lesions attributable to TVH have been detected only in the liver and pancreas. Livers generally are enlarged. Hepatic lesions consist of focal, gray, sometimes depressed areas up to several millimeters in diameter (Fig. 14.9). Lesion distribution is variable; birds that die usually exhibit very extensive lesions, which often coalesce and may be partially masked by vascular congestion and focal hemorrhage. Pancreatic lesions are less consistently observed than hepatic lesions. Lesions in the pancreas generally are roughly circular, gray-pink, and may extend across a lobe (Fig. 14.10).

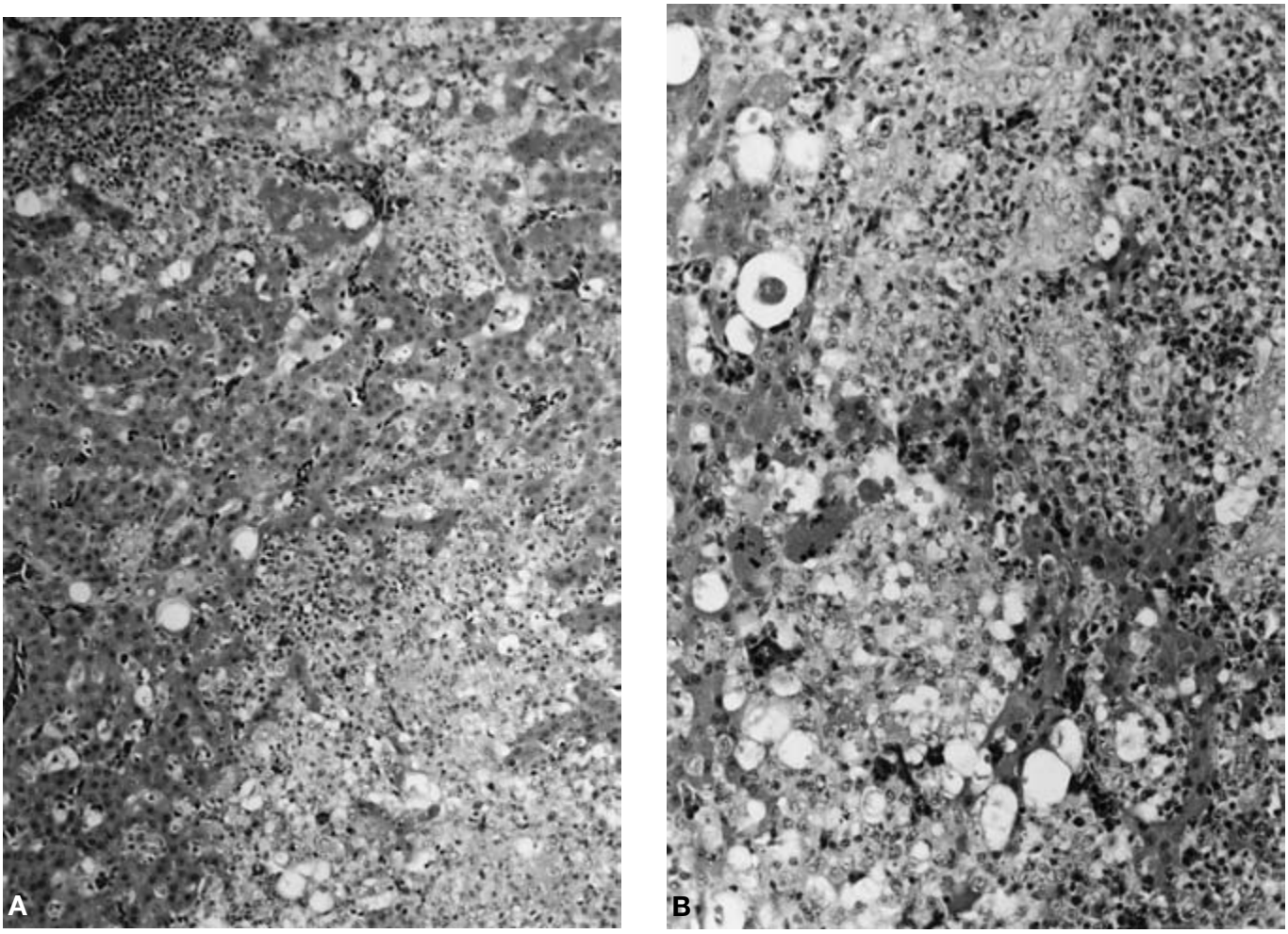
Microscopic

Vacuolation of hepatocytes occurs early in the course of infection with dense infiltration by mononuclear leukocytes, and proliferation of bile ductules. Lesions progress to overt focal necrosis with pooling of blood around the focus; necrotic cells are scattered among infiltrating lymphocytes (Fig. 14.11). Late in the course of infection lesions are composed of proliferating reticuloendothelial cells which frequently form giant cells (Fig. 14.11).

Pancreatic lesions exhibit the same general histopathologic changes as those observed in livers. Acinar cell degeneration and necrosis are observed with infiltration of macrophages and lymphocytes.

Immunity

Immunologic aspects of TVH have received little attention. Tzianabos and Snoeyenbos were unable to detect neutralizing antibodies in sera from recovered turkeys, or hyperimmunized chickens, turkeys and rabbits (12). However, immunity to rein-



14.11. Microscopic lesions of turkey viral hepatitis. (Barnes). A. Early lesions consist of multiple foci of vacuolar degeneration and coagulative necrosis. Cellular response primarily consists of lymphocytes and macrophages; heterophils are occasionally present but are not numerous. Pancreatic lesions are similar. In the liver, biliary hyperplasia generally is present, but the degree is highly variable among infected turkeys. B. As lesions mature, they advance along sinusoids, often investing islands of liver cells, creating an irregular margin.

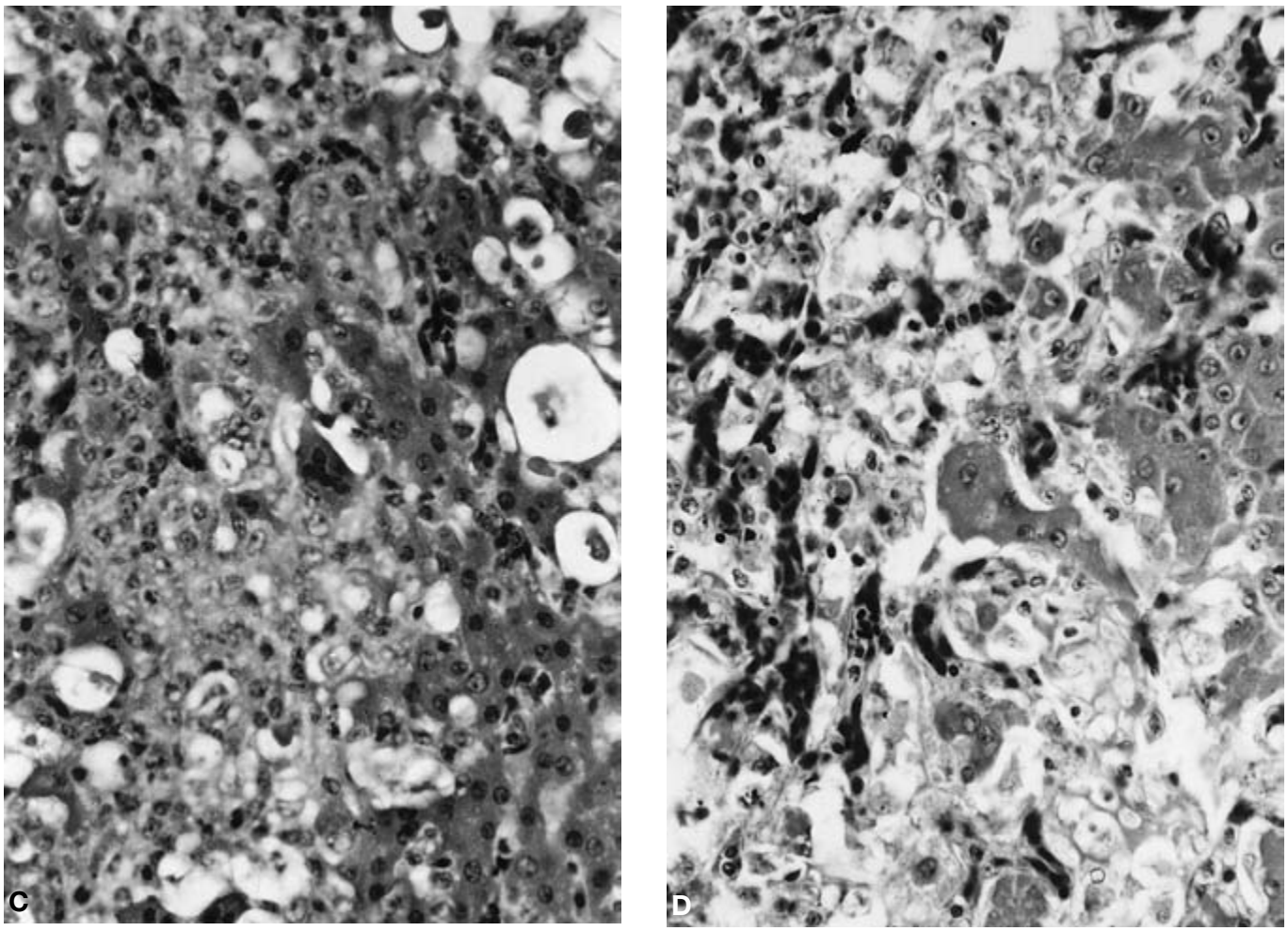
fection was observed in previously infected turkeys; reexposure of recovered birds after an interval of 21 days resulted in less frequent and less extensive lesions than in infected controls (9). Recovery from TVH results in resistance to reinfection but the duration of immunity has not been determined.

Diagnosis

Diagnosis of TVH may be based on histopathology or virus isolation; serological procedures currently are not available. Histopathology may be utilized to presumptively diagnose TVH, as the presence of lesions in both the liver and pancreas of turkeys is highly suggestive of the disease. However, similar lesions may be produced in the liver by a variety of bacterial, viral and protozoal agents. These include *Salmonella spp., Pasteurella multocida*, group I and group II avian adenoviruses (1, 14), reovirus (13) and *Histomonas meleagridis* (7, 8).

Isolation and Identification of Causative Agent

Virus isolation may be accomplished using a variety of tissues including liver, pancreas, spleen, kidney or feces, but liver is the preferred sample. Tissues or feces should be homogenized in an appropriate diluent such as minimal essential medium, and clarified by centrifugation; clarified fecal suspensions should be filtered through a 0.45-μm membrane filter. Homogenates of tissue or fecal suspensions are inoculated into 5- to 7-day-old embryonated chicken eggs by the yolk sac route. In TVH-positive cases, embryo mortality generally occurs 4–11 days PI (9). Embryo mortality is delayed if low virus titers are present and in some cases a second passage using yolk harvest may be required. Embryos exhibit cutaneous congestion and edema; dwarfing is observed in those embryos in which mortality is delayed and less cutaneous congestion is observed in these embryos (9). Liver lesions containing necrotic foci are sometimes observed in embryos that survive to 11 days PI. Embryonic fluids do not hemag-



14.11. (continued) C. Frequently, liver cells within or adjacent to lesions fuse together to form syncytial cells. D. Nuclear changes as seen here in hepatocytes adjacent to a lesion develop an appearance suggestive of inclusion bodies. Their nature is currently uncertain, but they are not believed to be of viral origin.

glutinate erythrocytes. Isolates may be further characterized by yolk sac or intraperitoneal inoculation of poults with yolk harvested from infected embryonated eggs; poults are examined for lesions 5–10 days PI.

Intervention Strategies

No specific therapeutic or prophylactic measures are available. Prevention of stress and other infections may be helpful in preventing normally subclinical disease from developing into TVH.

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Avian Encephalomyelitis

Bruce W. Calnek

Introduction

Avian encephalomyelitis (AE) is an infectious viral disease affecting young chickens, pheasants, quail, and turkeys. It is characterized by ataxia and rapid tremors, especially of the head and neck; because of the latter, it was often called "epidemic tremor."

No public health significance has been attached to this disease. The disease was of great economic importance to the commercial poultry industries prior to the widespread use of vaccines in the early 1960s.

History

Jones (47, 48) first encountered AE in 1930 in two-week-old commercial Rhode Island red chicks showing tremors. In 1931, two additional outbreaks were observed in one- and four-week-old chicks raised on different farms but originating from the same breeding flock. During the next two years, additional outbreaks were observed in Connecticut, Maine, Massachusetts, and New Hampshire, which led to AE being tagged "New England disease."

In 1934, Jones (48) reproduced the disease in susceptible chicks by intracerebral (IC) inoculation with filtrates of brain material from spontaneous cases. It was not until the mid-1950s, however, that Schaaf reported the first successful control of the disease by immunization (83). The epizootiology of AE was clarified by Calnek *et al.* in 1960 (21), and the development of an orally administered vaccine (22) soon followed. Historical accounts of the control of AE and other details were provided by Calnek (17), Tannock and Shafren (102), and van der Heide (107).

Etiology

Classification

Avian encephalomyelitis virus (AEV) is a member of the Picornaviridae family (53, 105) based on molecular characterization of the virus genome. Previous studies (13) suggested that AEV belongs to the *Enterovirus* genus, but based on the recent discovery that it has high levels of protein homologies with the hepatitis A virus (66, 105), it has been tentatively placed in the genus *Hepatovirus* (46).

The author wishes to acknowledge R.E. Luginbuhl and C.F. Helmboldt for their contributions to earlier editions of this chapter.

Morphology

Ultrastructure, Size, and Density

In purified preparations of AEV, Gosting *et al.* (33) observed virions with hexagonal profiles lacking envelopes. The virus was first shown to be filterable by Jones (48). Based on filtration studies, its size was found to range from 20–30 nm, or 16–25 nm, by Olitsky and Bauer (79) and Butterfield *et al.* (13), respectively. By electron microscopic (EM) examination of purified AEV, Gosting *et al.* (33) found the virions to be 24–32 nm in diameter; later EM studies by Tannock and Shafren (101) determined the mean diameter to be 26.1 ± 0.4 nm. Intracytoplasmic crystalline arrays observed in Purkinje cells from the brains of infected chickens had particles with diameters estimated to be 22 nm (23) or 25 nm (35).

The virus has a buoyant density of 1.31–1.33 g/mL (13, 33, 101) and a sedimentation coefficient of 148 S (33).

Symmetry

Gosting *et al.* (33) detected a fivefold symmetry with 32 or 42 capsomeres, in contrast to an earlier report by Krauss and Ueberschaer (55), who proposed an icosahedral symmetry with only 12 capsomeres.

Chemical Composition

Evidence that AEV is an RNA virus came from studies showing that viral replication *in vitro* was unaffected by DNase (8) or by a DNA inhibitor, 5-bromo-2N-deoxyuridine (93). Tannock and Shafren (102) initially detected four virus-specific proteins (VP 1–4) with molecular weights of 43,000, 35,000, 33,000, and 14,000, respectively. They noted in a later report (92), however, that one of the proteins actually was contaminating ovalbumin and that the other three VPs (1–3) were similar in size to those of poliovirus. They also reported that there were no differences between a field isolate and the embryo-adapted Van Roekel (VR) strain of virus when they were compared using a radioimmunoprecipitation assay, in keeping with earlier comparisons of physical, chemical, and serologic properties of the two types of viruses by Butterfield *et al.* (13).

Todd *et al.* (105) studied AEV RNA by reverse transcription-polymerase chain reaction and described the AEV genome as polyadenylated, single-stranded RNA, approximately 7.5 kb in size. Further definition of the virus composition came from Marvil *et al.* (66) who cloned and sequenced the complete RNA genome. They determined that it comprises 7032 nucleotides. Based on the projected product of a 6405-nucleotide open read-

ing frame starting at nucleotide 495, a close relationship (39% overall amino acid identity) with hepatitis A virus was established. One of the nonstructural proteins (2A) associated with AEV was found by Hughes and Stanway (38) to possess conserved motifs shared with two other picornaviruses, human parechoviruses, and Aichi virus. The authors noted that these motifs are characteristic of a family of cellular proteins, two of which are involved in the control of cell growth.

Susceptibility to Chemical and Physical Agents

AEV is resistant to chloroform, acid, trypsin, pepsin, and DNase and is protected against effects of heat by divalent magnesium ions (8, 13). The virus was found susceptible to a single exposure to formaldehyde fumigation (39). Beta-propriolactone inactivates the virus (12, 20).

Strain Classification and Pathogenicity

Although all isolates of AEV are serologically similar, there are two distinct pathotypes of virus. One, represented by natural field strains, is enterotropic. These strains infect chickens readily via the oral route and are shed in the feces. They are relatively nonpathogenic except in susceptible chicks infected by vertical transmission or by early horizontal transmission, in which case they cause neurologic signs. Neurologic disease also occurs following experimental infection by intracerebral inoculation of susceptible chickens.

Embryo-adapted strains constitute the other pathotype. These viruses are highly neurotropic and cause severe neurologic signs following intracerebral inoculation (invariable incidence) or parenteral routes such as intramuscular or subcutaneous inoculation (variable incidence). They do not infect via the oral route except with very high doses, and they do not spread horizontally (19, 44, 45, 70, 92, 113). Adaptation may occur after multiple passages in antibody-free chicken embryos (22, 69, 117), probably the result of selection of laboratory mutants (69). The most commonly used adapted strain is the VR strain, which had been passaged repeatedly by intracerebral inoculation of chickens (108). The VR strain already had the phenotype of adapted strains when first inoculated into embryos after 150 chicken passages (18, 98).

Both pathotypes can replicate in embryos derived from a susceptible flock, but natural strains do not cause obvious signs or gross lesions. However, adapted strains are pathogenic for embryos, causing muscular dystrophy (Fig. 14.12) and immobilization of skeletal muscles (18, 50). The virus was detected in brains of inoculated embryos 3–4 days postinoculation (PI), and peak titers were found 6–9 days PI (10, 18). Histopathologic changes in embryos infected with egg-adapted virus have been described as uniform in character but variable in intensity and location and consisting of encephalomalacia and muscular dystrophy (50). Muscular changes consisted primarily of eosinophilic swelling and necrosis, fragmentation and loss of striations of affected fibers with rare sarcolemmal proliferation and heterophil infiltration. Neural lesions were characterized by severe local edema, gliosis, vascular proliferation, and pyknosis.



14.12. Chicken embryos on the right were inoculated via the yolk sac with the Van Roekel strain of avian encephalomyelitis virus on the 6th incubation day. Control embryos are on the left. The affected embryos, examined on the 18th incubation day, show extreme muscular dystrophy (most evident in the embryo with the skin removed) and rigidity of the legs.

Laboratory Host Systems

Virus may be propagated in the baby chick, chicken embryos from susceptible flocks, and a variety of cell culture systems. Chicks and embryos must be from a susceptible flock except in the case of intracerebral inoculation of chicks. Several routes of inoculation in embryos have been used (50, 98, 117), but inoculation via the yolk sac at 5–7 days of embryonation generally is considered the method of choice. Gross lesions (see previous section) are observed only with adapted strains. Tannock and Shafren (102) reviewed numerous reports on cell-culture propagation of AEV, beginning with the first successful replication of the VR strain of AEV in chicken embryo brain cultures in 1967 by Mancini and Yates (63). Subsequently, fibroblasts, kidney cells, and neuroglial cells from chicken embryos and pancreatic cells from young chicks were used to cultivate both adapted and field strains of virus (3, 51, 54, 64, 65, 76, 86). Titers, particularly with natural strains, were generally low (rarely exceeding $10^{3.5}$ EID_{50}/mL), and cytopathic effects have not been described. Replication in cell cultures is detected by inoculation of embryos (adapted strains only) or by tests for antigen using immunofluorescence or enzyme-linked immunosorbent assays (ELISA). Nicholas *et al.* (75) suggested that chicken embryo neuroglial cells may provide an excellent substrate for production of AEV antigen suitable for serologic tests, such as immunodiffusion and ELISA, and they recommended that cell cultures be adopted as the method of choice for titration of AE vaccine (75). Shafren and Tannock (92) compared the VR strain, a field isolate, and a vaccine strain for the ability to grow in chicken embryo brain cultures; titers with the VR strain, after a 2-day eclipse, were 8–10 times higher than those with the other strains, and virus was largely cell associated. Abe (1) failed to demonstrate replication of AEV in a variety of established mammalian cell lines.

Pathobiology and Epidemiology

Incidence and Distribution

Avian encephalomyelitis occurs virtually worldwide (102, 107). Nearly all chicken flocks eventually become infected with the virus, but the incidence of clinical disease is very low unless a breeder flock is not vaccinated and becomes infected after the commencement of egg production. Turkey flocks apparently also experience high rates of natural infection based on serological surveys (25, 26). The rate of infection in pheasants and quail is not known.

Natural and Experimental Hosts

Avian encephalomyelitis virus has a limited host range. Chickens, pheasants, coturnix quail, pigeons and turkeys have all succumbed to naturally occurring infection (106, also see reviews 11, 107). Experimental infection of young quail chicks (34) caused clinical signs, and the infection spread to breeding quail in the same room. Infection of the adults resulted in reduced egg production and hatchability, and clinical AE developed in chicks hatched from eggs laid during the outbreak. The naturally occurring disease in turkeys is essentially the same as that in chickens (37). Ducklings, poults, young pigeons, and guinea fowl also have been infected experimentally. Mice, guinea pigs, rabbits, and monkeys were refractory to virus introduced intracerebrally (67, 74, 80, 109, 110). Van Steenis (112) found naturally occurring AEV antibodies in serums from partridge, pheasant, and turkeys but not in serums from finches, sparrows, starlings, pigeons, jackdaws, rooks, doves, or ducks. The latter four species also failed to develop antibodies after oral exposure to AEV. AEV antibodies were reported to be present in sera from ostriches (14), waved albatrosses (81) and penguins (52). Bodin *et al.* (4) compared adult pheasants and red and gray partridges for sensitivity to intramuscular or oral-nasal inoculation with the VR strain of virus. All became infected, but the severity of disease based on signs and lesions was greatest in gray partridges and least in pheasants. Embryonated eggs from the three species were also susceptible to infection.

Transmission

The IC route of inoculation has given the most consistent results in reproducing AE in chickens. Other routes by which infection has been experimentally established are intraperitoneal, subcutaneous, intradermal, intravenous, intramuscular, intrasciatic, intraconjunctival sac, oral, and intranasal inoculation (13, 21, 27, 49, 78, 89, 109).

Under natural conditions, AE is essentially an enteric infection (21). Ingestion is the usual portal of entry (21, 36); exposure via the respiratory tract may be unimportant other than through the coincident exposure of the alimentary tract (21). Virus is shed in the feces for a period of several days, and because it is quite resistant to environmental conditions, it remains infectious for long periods of time. The period during which virus is excreted in feces is dependent in part on the age of the bird when infected. Very young chicks may excrete virus for more than two weeks, whereas those infected after three weeks of age may shed virus for only about five days (116). Shafren and Tannock (91) found virus in feces from 4–10 days after exposure to a field strain of AEV. Infected litter is a source of virus easily transmitted horizontally by tracking or fomites. Infection spreads rapidly from bird to bird within a pen or house once introduced and from pen to pen on farms where no special precautions are taken to prevent spread. Birds in isolated flocks of a single age group were found to be less likely to have encountered infection than chickens on farms with multiple-age groups. Virus spread was found to be less rapid among birds in cages than in those on the floor (21, 28, 90).

Vertical transmission is a very important means of virus dissemination, based on both field evidence and experimental results (21, 49, 89, 104, 111). Taylor and Schelling (103) reported that 57% of breeder flocks tested in North America had been exposed to the virus by 5 months of age; however, 96% were serologically positive by 13 months. Although the source of infection for susceptible flocks is unknown, it is likely that it is carried from infected farms by people or fomites. When susceptible flocks are exposed after sexual maturity, the hens infect a variable proportion of their eggs. Calnek *et al.* (21) showed that infected embryos and chicks came from eggs laid during the period 5–13 days after experimental infection of susceptible breeders. Jungherr and Minard (49) reported that hatchability of eggs from an infected flock was not affected. Conversely, Taylor *et al.* (104) observed a high embryo death pattern during the last three days of incubation. The percentage of embryos that hatched declined from a 78.6% preinfection level to 59.6% during the clinical stage and increased to 75.4% postinfection. Eggs produced just prior to and during the period of depressed egg production showed decreased hatchability and increased embryo mortality during the last three days of incubation. Furthermore, only chicks from the group with depressed hatchability showed signs of AE; chicks hatched prior to and after the affected hatch appeared normal. Similar observations have been reported by other workers (21, 84).

Calnek *et al.* (21) demonstrated that virus transmission can occur in the incubator. Chicks hatched from eggs inoculated at six days' incubation manifested signs on the first day of age; by the sixth day, 49 of 52 showed clinical evidence of AE. Chicks from uninoculated eggs hatched with the infected birds first manifested signs on the tenth day, and 15 of 18 chicks developed clinical signs. An isolated control group of 19 chicks remained negative.

The possibility of a carrier status is unknown. Richey (84) incriminated a ready-to-lay pullet flock housed in the same building, but in a separate pen, as the source of infection for outbreaks that occurred in several susceptible breeding flocks at 45 weeks of age. The pullet flock had experienced an acute outbreak of AE at 3 weeks of age; it was suggested that a carrier existed in the flock. Although certain aspects of transmission have been well established, other phases remain unknown.

Incubation Period

Studies conducted by Calnek *et al.* (21) demonstrated that the incubation period in chicks infected by embryo transmission was 1–7 days, whereas chicks infected by contact transmission or oral administration had a minimum incubation period of 11 days.

Clinical Signs

Avian encephalomyelitis presents an interesting syndrome. In naturally occurring outbreaks, it usually makes its appearance when chicks are 1–2 weeks of age, although affected chicks have been observed at the time of hatching. Affected chicks first show a slightly dull expression of the eyes, followed by a progressive ataxia from incoordination of the muscles, which may be detected readily by exercising the chicks. As the ataxia grows more pronounced, chicks show an inclination to sit on their hocks. When disturbed, they may move about, exhibiting little control over speed and gait; finally, they come to rest or fall on their sides. Some may refuse to move or may walk on their hocks and shanks. The dull expression becomes more pronounced and is accompanied by a weakened cry. Fine tremors of the head and neck may become evident, the frequency and magnitude of which may vary. Exciting or disturbing the chicks may bring on the tremor, which may continue for variable periods and recur at irregular intervals. Ataxic signs usually, but not always, appear before the tremor. In some cases, only tremor has been observed. Ataxia usually progresses until the chick is incapable of moving about, and this stage is followed by inanition, prostration, and finally death. Chicks with marked ataxia and prostration are frequently trampled by their penmates. Some chicks with definite signs of AE may survive and grow to maturity, and in some instances signs may disappear completely. Survivors may later develop blindness from an opacity giving a bluish discoloration to the lens (7, 82).

There is a marked age resistance to clinical signs in birds exposed after they are 2–3 weeks of age (see "Pathogenesis of the Infectious Process"). Mature birds may experience a temporary drop in egg production (5–10%) but do not develop neurologic signs.

Morbidity and Mortality

Morbidity from the naturally occurring disease has been observed only in young stock. The usual morbidity rate is 40–60% if all the chicks come from the infected flock. Mortality averages 25% and may exceed 50%. These rates are considerably lower if many of the chicks comprising the flock originate from breeder flocks of immune birds.

Pathology

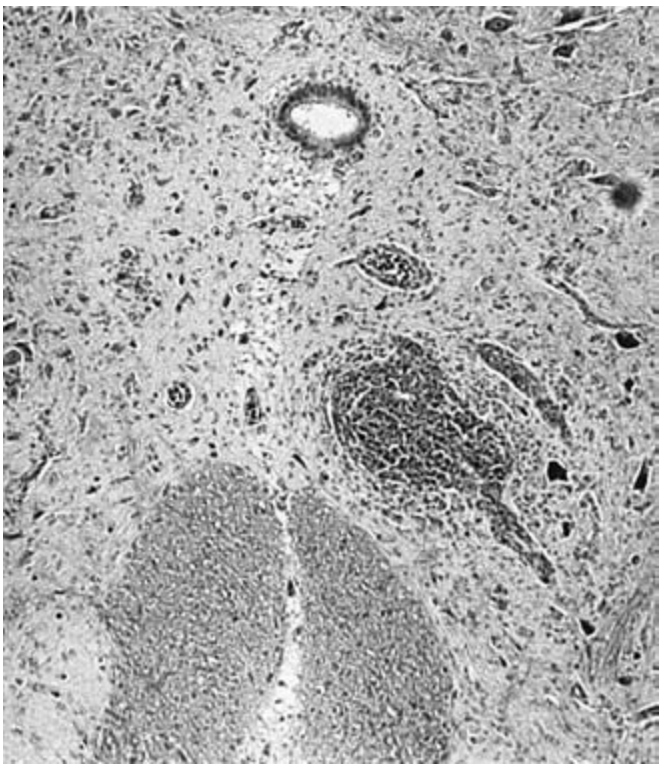
Gross

The only gross lesions associated with AE in chicks are whitish areas (due to masses of infiltrating lymphocytes) in the muscularis of the ventriculus. These are subtle changes and require favorable conditions to be discerned. No changes have been described for infected adult birds, other than the lens opacities described in "Signs."

Microscopic

The principal changes are in the CNS and some viscera. The peripheral nervous system is not involved—a point of importance in differential diagnosis.

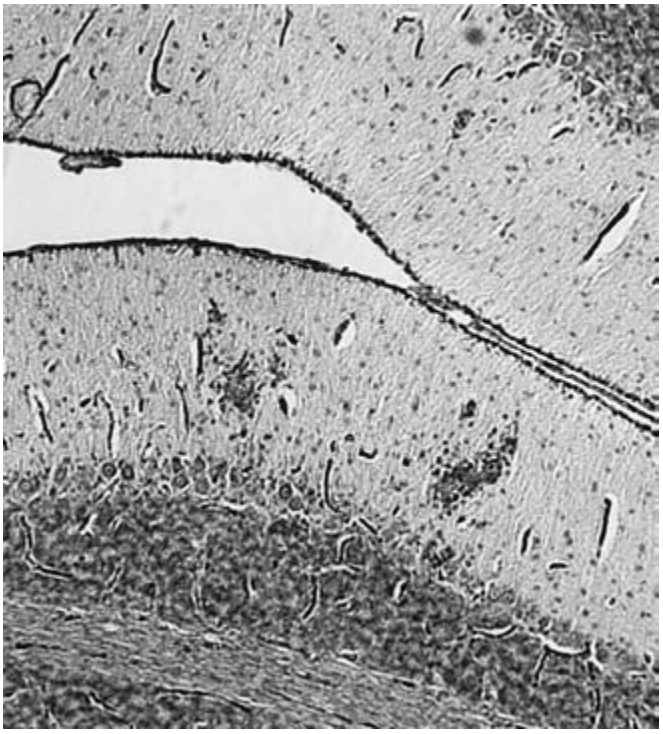
In the CNS, the lesions are those of a disseminated, nonpurulent encephalomyelitis and a ganglionitis of the dorsal root ganglia. The most frequently encountered addition is a striking perivascular infiltrate seeming to occur in all portions of the brain and spinal cord (Figs. 14.13 and 14.14), except the cerebellum, where it is confined to the nucleus (n.) cerebellaris. Infiltrating small lymphocytes may pile up several layers to form an impressive perivascular cuff.



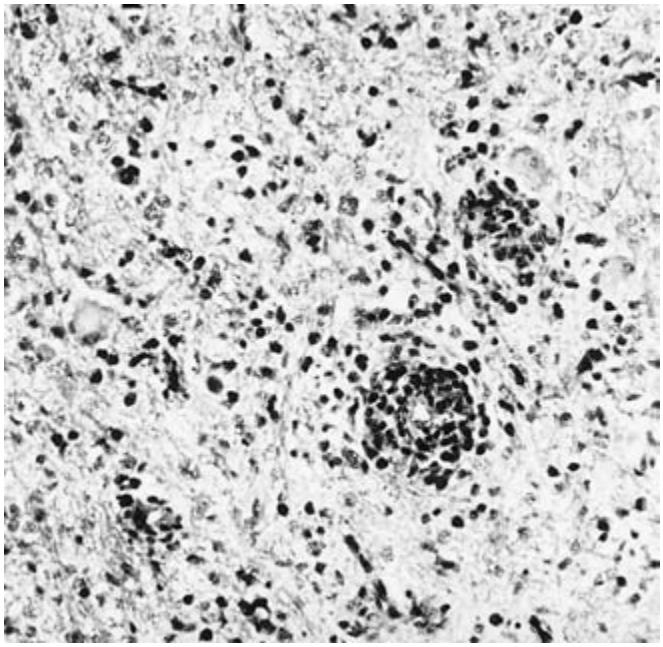
14.13. Spinal cord at lumbar level of chick. Large glial nodule and several perivascular infiltrates of lymphocytes are in gray matter. Central canal is at top. H & E, ×75.

Microgliosis occurs as diffuse and nodular aggregates. The glial lesion is seen chiefly in the cerebellar molecular layer, where it tends to be compact (Fig. 14.15). A loose gliosis usually is found in the n. cerebellaris, brain stem, midbrain, and optic lobes and less often in the corpus straita. In the midbrain, two nuclei, Cn. rotundus and n. ovoidalis, are invariably affected with a loose microgliosis that can be considered pathognomonic. Another lesion of pathognomonic significance is central chromatolysis (axonal reaction) of the neurons in the nuclei of the brain stem, particularly those of the medulla oblongata (Fig. 14.16). If several sagittal sections are made, one can almost always find this alteration. The dying neuron is surrounded by satellite oligodendroglia, and, later, microglia phagocytize the remains; the central chromatolysis is never seen without an attending cellular reaction.

Hishida *et al.* (35) examined brain and spinal cord lesions from experimentally infected chicks on a sequential basis using light- and electron-microscopy and immunofluorescence techniques. They considered the most characteristic changes to be degeneration of Purkinje neurons in the cerebellum and motor neurons in the medulla oblongata and spinal cord. The central chromatolysis



14.14. Perivascular infiltration and gliosis are seen in the nucleus cerebellaris. H & E, ×363. (Jakowski)



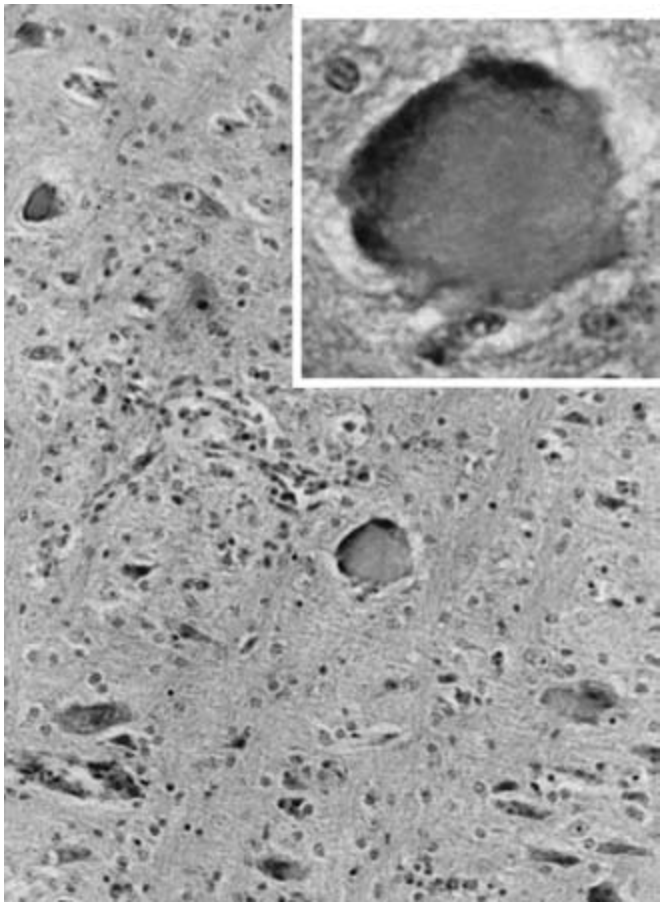
14.15. Cerebellum of chick. Glial foci common in avian encephalomyelitis are in the molecular layer. H & E, ×375.

observed in the motor neurons was thought to be reversible, whereas affected Purkinje neurons always became necrotic. Purkinje neurons contained abundant viral antigen and cystalline arrays of virus particles in the cytoplasm, confirming the observations of Cheville (23). Degenerated neuronal cells showed dilatation of rough-surfaced endoplasmic reticulum, a reduction in ribosomes, and mitochondrial degeneration (23, 35, 119). Liu *et al.* (56, 57, 58), in a series of studies on the nonstructural virus protein 3A, have shed some light on the subject of cell death due to AEV infection. They showed that this membrane-interacting protein can result in membrane permeability and is capable of inducing apoptosis by activating the cytochrome c/capase-9 pathway.

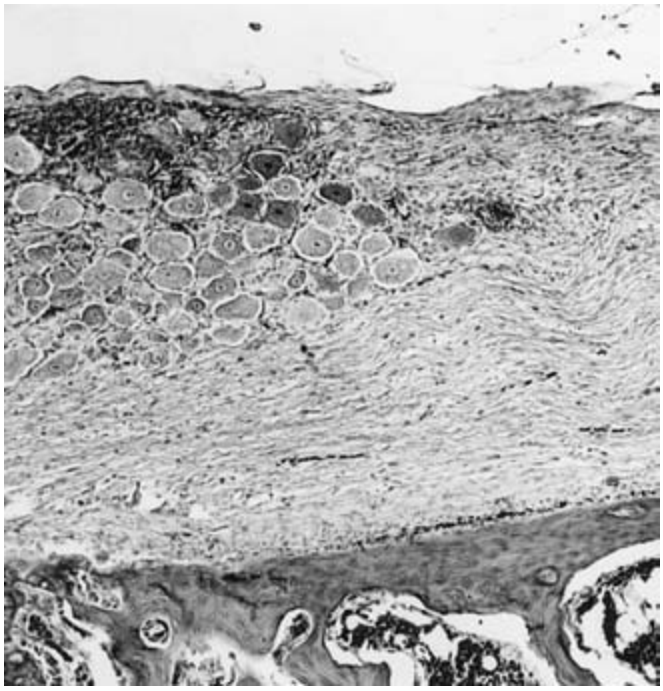
The dorsal root ganglia often contain rather tight aggregates of small lymphocytes amid the neurons. The lesion is always confined to the ganglion and never enters the nerves (Fig. 14.17).

In general, signs cannot be correlated with severity of lesions or distribution in the CNS.

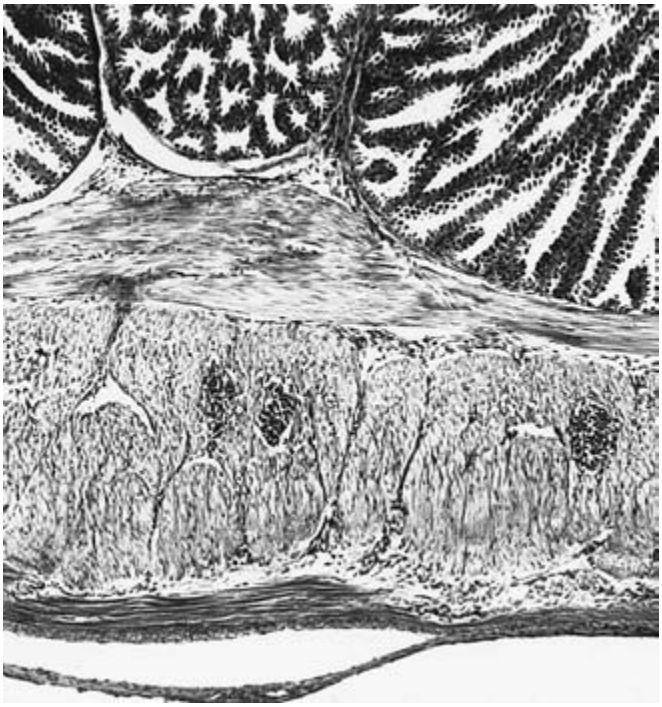
Visceral lesions appear to be hyperplasia of the lymphocytic aggregates scattered in a random fashion throughout the bird. In the proventriculus, aggregates of a few small lymphocytes normally are within the muscular wall; in AE, these are obvious dense nodules that are certainly pathognomonic (Fig. 14.18). Similar lesions occur in the ventriculus muscle, but unfortunately, they also occur in Marek's disease. In the pancreas, circumscribed lymphocytic follicles are normal (59), but in AE the number increases several times (Fig. 14.19). In the myocardium and particularly the atrium, aggregates of lymphocytes are con-



14.16. Medulla oblongata of chick. There is diffuse gliosis, and in the center a neuron is undergoing central chromatolysis. H & E, ×375. Inset shows tigrolysis and loss of nucleus, ×3480.



14.17. Dorsal root ganglion of lumbar level of chick. Dense infiltrate of lymphocytes is confined to ganglion. Sciatic nerve is unaffected. H & E, ×375.



14.18. Proventriculus of chick. Dense lymphocytic foci are in muscular wall. This lesion is pathognomonic. H & E, ×330.

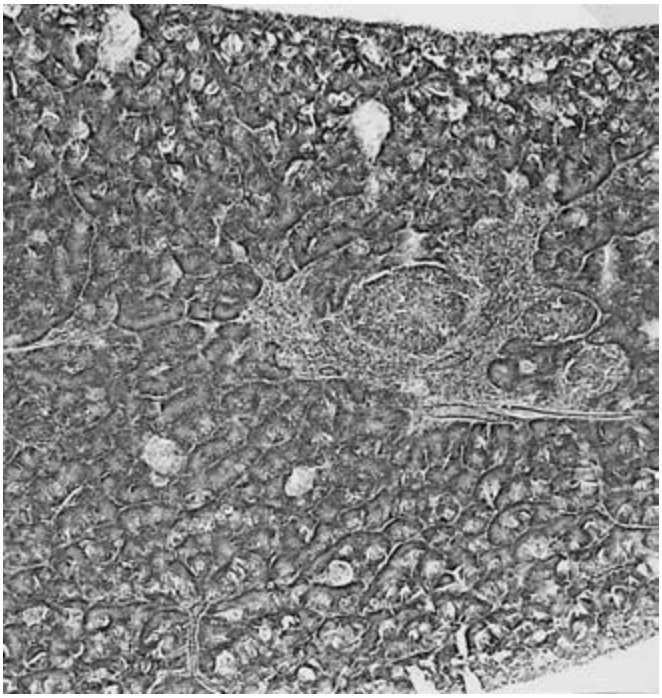
sidered to be the result of AE (97). Lymphocytes in the myocardium of young chicks are not unusual; however, one may consider them a lesion only if they are widespread and accompanied by previously noted alterations.

There appears to be an excellent correlation between clinical signs and histologic lesions in the nervous system. In one study, 11% had signs but no lesions, and 8% had lesions but no signs (49). Later, Jungherr believed that all birds with clinical signs had histologic lesions. This was based on more intensive research that, in turn, was based on multiple sections of brain and viscera. Experimentally inoculated chicks killed in sequential fashion invariably yield lesions 1–2 days before clinical signs. Recovered birds free from signs have CNS lesions for at least one week and probably much longer.

Pathogenesis of the Infectious Process

Significant differences exist between embryo-adapted AEV and field strains of the virus in terms of pathogenesis. This is largely because the adapted strains generally lose the enterotropic properties that characterize the natural strains. Consequently, adapted strains are relatively noninfectious by the oral route of exposure, do not replicate in the intestine, and are not excreted in the feces following infection by parenteral inoculation (19, 21; *see also* 102).

Localization of viral antigen using virus isolation, immunodiffusion, immunofluorescence, and ELISA techniques has been reported by van der Heide (107), Braune and Gentry (6), Ikeda and coworkers (40, 42, 45), Miyamae and coworkers (68, 70, 71, 72, 73), and Shafren and Tannock (91, 92). In young chicks exposed



14.19. Pancreas of young chick. Several follicles of lymphocytes are present. This lesion is significant only when abnormal numbers of follicles are present. H & E, ×330.

orally to field strains of AEV, primary infection of the alimentary tract, especially in the duodenum, is rapidly followed by a viremia and subsequent infection of the pancreas and other visceral organs (liver, heart, kidney, spleen) and skeletal muscle, and finally the central nervous system (CNS). Alimentary tract infections involve muscular layers, and pancreatic infections are found in both the acinar and islet cells, persisting more in the latter. Viral antigen is relatively abundant in the CNS where Purkinje neurons and the molecular layer of the cerebellum are apparently favored sites of virus replication. Neuroglial cells are probably also infected given the report of their susceptibility to AEV *in vitro* (75). Chicks with clinical signs at 10–30 days of age tend to have viral antigen mostly in the CNS and pancreas; lesser amounts of antigen have been seen in heart and kidney; and only very small amounts have been seen in liver and spleen. Persistence of the virus infection is common in the CNS, alimentary tract, and pancreas. Interestingly, the CNS and the pancreas are the only sites uniformly infected by embryo-adapted strains of AEV, although small amounts of virus may be found transiently in other tissues including the liver, heart, and spleen.

Van der Heide (107) was unable to find viral antigen when tissues from experimentally infected mature birds were examined. However, Miyamae (71) did detect viral antigen in viscera and intestinal tract of two-year-old hens infected orally with field strains of AEV. In the intestinal tract, viral antigen was found in the epithelial tunica mucosa, circular muscle layer, and/or muscularis mucosa and in the tunica propria mucosa, but the detection rate was lower than has been reported for young chicks. No viral antigen was found in the CNS; presumably this lack of infection correlates with the absence of clinical disease in infected adults. As in young chicks, infection of older birds with embryo-adapted AEV has a more limited tissue distribution and/or lower titers of AEV in tissues other than those of the CNS, when compared with infection with field strains (42, 43).

Cheville (23) and Westbury and Sinkovic (113, 114, 115, 116) did much to clarify certain aspects of the pathogenesis of the naturally occurring or experimental disease. Age at exposure was especially important; Cheville noted that birds infected at 1 day of age generally died, whereas those infected at 8 days developed paresis but usually recovered, and infection at 28 days caused no clinical signs. Bursectomy but not thymectomy abrogated the age resistance. Westbury and Sinkovic (113) also noted disease when infection was initiated at 14 or fewer days of age, but not when it occurred at 20 or more days. They confirmed Cheville's (23) conclusion that humoral immunity was the basis of age resistance. In their studies, they correlated young age (thus, immunologic incompetence) with extended viremia, persistence of virus in the brain, and development of clinical disease. Presumably, the immune response of an immunologically competent bird would stop the spread of infection before it reached the CNS. Age resistance was not expressed when experimental infection was induced by IC inoculation of virus. Interestingly, Calnek *et al.* (21) found clinical signs in contact-exposed young chicks to have a minimum incubation period of 10–11 days, the same time that virus-neutralizing (VN) antibodies can be detected in adult birds.

Immunity

Birds recovered from naturally occurring and experimental infection develop circulating antibodies capable of neutralizing the virus (see reviews 11, 16, 15, 102).

Cheville (23), and later Westbury and Sinkovic (114), clearly showed that humoral, but not cellular, immunity was important in curtailing infection. If the response is rapid, as is usual in birds greater than 21 days of age, the CNS infection apparently does not progress to the point where clinical signs may develop.

Active

When chickens are immunologically competent, the serologic response can be relatively rapid. Data from Calnek *et al.* (21) suggested that chicks from eggs laid as early as 11 days after exposure already carried passively acquired antibodies, because they were resistant to contact exposure after hatching. Also, positive VN tests (i.e., those with a neutralization index (NI) of 1.1 or greater) (18), can be found after 11–14 days PI (22, 115), and positive immunodiffusion (ID) tests as early as 4–10 days PI (41).

Flocks of chickens with positive serology rarely if ever have recurrent outbreaks of AE.

Passive

Antibodies are transferred to progeny from the dam via the embryo and can be demonstrated in egg yolk (99). Birds from immune dams were not fully susceptible to oral inoculation until 8–10 weeks of age, and antibodies were demonstrated in the serum until 4–6 weeks of age (22). Passively acquired antibodies can prevent development of disease (116) and prevent or reduce the period of virus excretion in feces (21, 116). They also render embryonating eggs resistant to virus inoculated via the yolk sac, forming the basis for the embryo-susceptibility test (see "Diagnosis").

Diagnosis

Isolation and Identification of Causative Agents

The brain is an excellent source of virus for isolation, although other tissues and organs induce the disease when injected into chicks (48, 108). Miyamae (72) found that in addition to the brain, the pancreas and duodenum were especially reliable sources of virus.

The need to titrate vaccine virus makes a sensitive method for virus detection very important. One system for assay of virus is to inoculate embryos (obtained from a susceptible flock) via the yolk sac when 5–7 days of age, allow these to hatch, and observe chicks for signs of disease during the first 10 days (9, 36). When clinical signs appear, brain, proventriculus, and pancreas should be examined for lesions as described in "Pathology." Additionally or alternatively, brain, pancreas, and duodenum from affected chicks can be examined for specific viral antigen by immunofluorescence (5, 6, 68, 70, 107) or ID (40) tests. A newly described monoclonal antibody (74), which recognizes a common epitope among AEV strains, may be a useful addition to the reagents available for virus detection in vaccine titrations or for other assays.

Berger (3) infected chicken embryo brain cell cultures and then used an indirect fluorescent antibody (FA) test to detect viral antigen. He found it to be more sensitive than the embryo inoculation method. Nicholas *et al.* (76) compared several methods for detection of AEV. Inoculation of brain cell cultures followed by indirect FA test was found to be convenient, but inoculation of two-week-old susceptible chicks followed by serologic tests such as ELISA or ID was slightly more sensitive. Xie *et al.* (118) developed a reverse transcriptase-polymerase chain reaction to detect AEV and found it to be both specific and sensitive to as little as 10 picograms of AEV RNA.

Serology

Chickens exposed to AEV develop antibodies that can be measured with the standard VN test (18, 99), indirect FA test (24), the ID test (31, 41, 60), ELISA (29, 95, 120 *see also* 96), and passive hemagglutination test (2).

The VR embryo-adapted strain is recommended to determine the neutralizing capacity of the serum or plasma. Six-day-old embryos inoculated via the yolk sac with virus dilutions mixed with serum are examined for characteristic lesions 10–12 days PI. An NI of 1.1 or greater is considered as positive evidence of previous exposure to AEV. Among samples from a recently exposed flock, the NI may vary from 1.5 to 3.0. Antibodies may be detected as early as the second week after exposure and remain at significant levels for at least several months. Calnek and Jehnich (18) reported that in many instances birds having no detectable VN antibodies (NI less than 1.1) would resist IC challenge with as many as 10,000 EID_{50} of virus.

Another method to determine immunity of a flock is the embryo susceptibility (ES) test (99). Fertile eggs from the flock to be tested are incubated, along with control eggs from a known susceptible flock. After six days, each embryo is inoculated via the yolk sac with 100 EID_{50} of egg-adapted virus. Embryos are examined 10–12 days PI for characteristic lesions. If 100% of embryos are affected, the flock is considered susceptible; less than 50% affected indicates immunity. Intermediate figures should be considered nondefinitive and may indicate recent exposure.

Titers in the indirect FA test appear to parallel those of the VN test. Choi and Miura (24) and Dovadola *et al.* (26) found the indirect FA test to be as useful as the ES test for assessing immunity in turkey breeder flocks.

Standard procedures for ID tests were first reported by Ikeda (40, 41), who used concentrated tissue extracts from infected embryos as the antigen. Antibodies could be found as early as 4–10 days post-exposure, and these persisted for at least 28 months. Rare false-positives and false-negatives were reported when the ID test was compared with the VN test. Girshick and Crary (31), who used a similar antigen, confirmed Ikeda's general results but did not find discrepancies between the ID and VN tests.

Ahmed *et al.* (2) described a passive hemagglutination test, which they found to be more sensitive than the ID test and equal to the ES test in sensitivity.

An ELISA test using purified viral antigen compared well with the VN test and was found more suitable than the ID test for evaluation of immunity (29, 62, 85, 100). The use of a negative-antigen-subtraction step may enhance the ability to discriminate between positive and negative sera (91). Smart *et al.* (96) determined ELISA to correlate well with the ES test. They used ELISA to diagnose active infections with AEV by an increase in titer with sequential serum samples. Garrett *et al.* (30) were able to correlate ELISA titers in hens with the resistance of progeny embryos to challenge with AEV.

Differential Diagnosis

In spontaneous cases, a tentative and frequently definite diagnosis of disease can be made when a complete history of the flock and typical specimens are provided for histopathology.

Histopathologic evidence of gliosis, lymphocytic perivascular infiltration, axonal type of neuronal degeneration in the CNS, and hyperplasia of the lymphoid follicles in certain visceral tissues usually can be considered as a basis for a positive diagnosis. Virus isolation or a rise in titer with serologic tests gives a more specific diagnosis.

Avian encephalomyelitis should not be confused with other avian diseases manifesting similar clinical signs, such as Newcastle disease, equine encephalomyelitis infection, nutritional disturbances (rickets, encephalomalacia, riboflavin deficiency), and Marek's disease.

Avian encephalomyelitis is predominantly a disease of one- to three-week-old chicks. Because Newcastle disease may strike at this time, a problem of differential diagnosis can arise. Certain histological lesions are peculiar to AE: central chromatolysis as opposed to peripheral chromatolysis of Newcastle disease, gliosis in the n. rotundus and n. ovoidalis that is not observed in Newcastle disease, lymphocytic foci in the muscular wall of the proventriculus, and circumscribed lymphocytic follicles in the pancreas. Newcastle disease rarely causes an interstitial pancreatitis.

Encephalomalacia generally appears 2–3 weeks later than AE, and from the standpoint of clinical history, the signs should be no problem. Histologically, it causes severe degenerative lesions in no way similar to AE.

Marek's disease, which occurs still later, presents little difficulty. The peripheral nerve involvement and state of lymphomatosis of the viscera are two criteria not seen in AE.

Intervention Strategies

No satisfactory treatment is known for acute outbreaks in young chicks. Removal and segregation of affected chicks may be indicated under certain conditions, but they generally will not develop into profitable stock. After a flock has experienced an outbreak of AE, no further evidence of it is likely to be observed (89).

Vaccination

Control of AE is achieved by vaccination of breeder flocks during the growing period to ensure that they do not become infected after maturity, thereby preventing dissemination of the virus by the egg-borne route. Also, maternal antibodies protect progeny against contact to AEV during the critical first 2–3 weeks. Vaccination may also be used with commercial egg-

laying flocks to prevent a temporary drop in egg production associated with AE. Vaccines used to control AE in chickens have been shown to be efficacious in turkeys as well (25).

The development of AE vaccination strategies has been detailed by Calnek (17). Inactivated vaccines have been developed (12, 20, 61, 88) and may be useful in flocks already in production or where the use of a live virus is contraindicated. Most flocks, however, are vaccinated with a live, embryo-propagated virus, such as strain 1143 (22), which can be administered by naturally occurring routes such as via drinking water or by spraying (9, 22, 28). Live virus vaccines, which can be stored frozen or after lyophilization (8, 83), are similar to field virus in that they spread readily within a flock. This allows for administration per os to a small percentage of the birds in a flock, which then spread infection to others, although this method is generally unsatisfactory for birds in wire cages (28, 90). Shafren *et al.* (94) found that serologic responses to vaccine administered conjunctivally to 10% (but not 5%) of a flock were as good as those following drinking-water administration of virus to the entire flock. Vaccination by wing-web inoculation of AEV is also practiced in many flocks, but this method may carry some risk of clinical signs (32). Generally, vaccination is done after 8 weeks of age and at least 4 weeks before egg production.

It is very important that embryo adaptation of strains used for live virus vaccines does not occur because 1) adapted virus loses its ability to infect via the intestinal tract and is, therefore, no longer efficacious when administered by naturally occurring routes (22); and 2) adapted virus, like field strains, can cause clinical disease when administered by the wing-web route (19). Glisson and Fletcher (32) observed clinical encephalitis in broiler-breeder pullets given embryo-propagated AEV vaccine by the wing-web route and concluded that the most probable explanation was that the vaccine virus was inadvertently adapted during manufacture. Adaptation is detected by careful monitoring of inoculated embryos used in the production of vaccine for characteristic signs (see "Etiology"), and any adapted virus can be eliminated from vaccine seed virus stocks by passage in susceptible chicks inoculated orally.

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Avian Hepatitis E Virus Infections

X. J. Meng, H. L. Shivaprasad, and C. Payne

Introduction

Hepatitis-splenomegaly (HS) syndrome is a disease of layer and broiler breeder chickens characterized by increased mortality and decreased egg production and is primarily caused by avian hepatitis E virus (avian HEV)(19, 40, 42, 51). Dead birds have red fluid or clotted blood in their abdomens, and enlarged livers and spleens. Field veterinarians have recognized this disease since the mid 1980's in some parts of the United States. Although first described as HS syndrome, the disease is also referred to as big-liver-and-spleen (BLS) disease (30, 38–39, 58), necrotic hemorrhage hepatitis-splenomegaly syndrome (45), necrotic hemorrhagic hepatomegalic hepatitis (55), hepatitis-liver hemorrhage syndrome (25), and chronic fulminating cholangiohepatitis (26). Since there are only a few reports of HS syndrome outbreaks in Canada and the United States, the economic impact of this disease is not known. However, in Australia the disease was considered the most economically significant disease of broiler breeders with estimated annual loss at 8 eggs per hen in 50% of broiler breeder flocks for approximately 2.8 million Australian dollars (39–41).

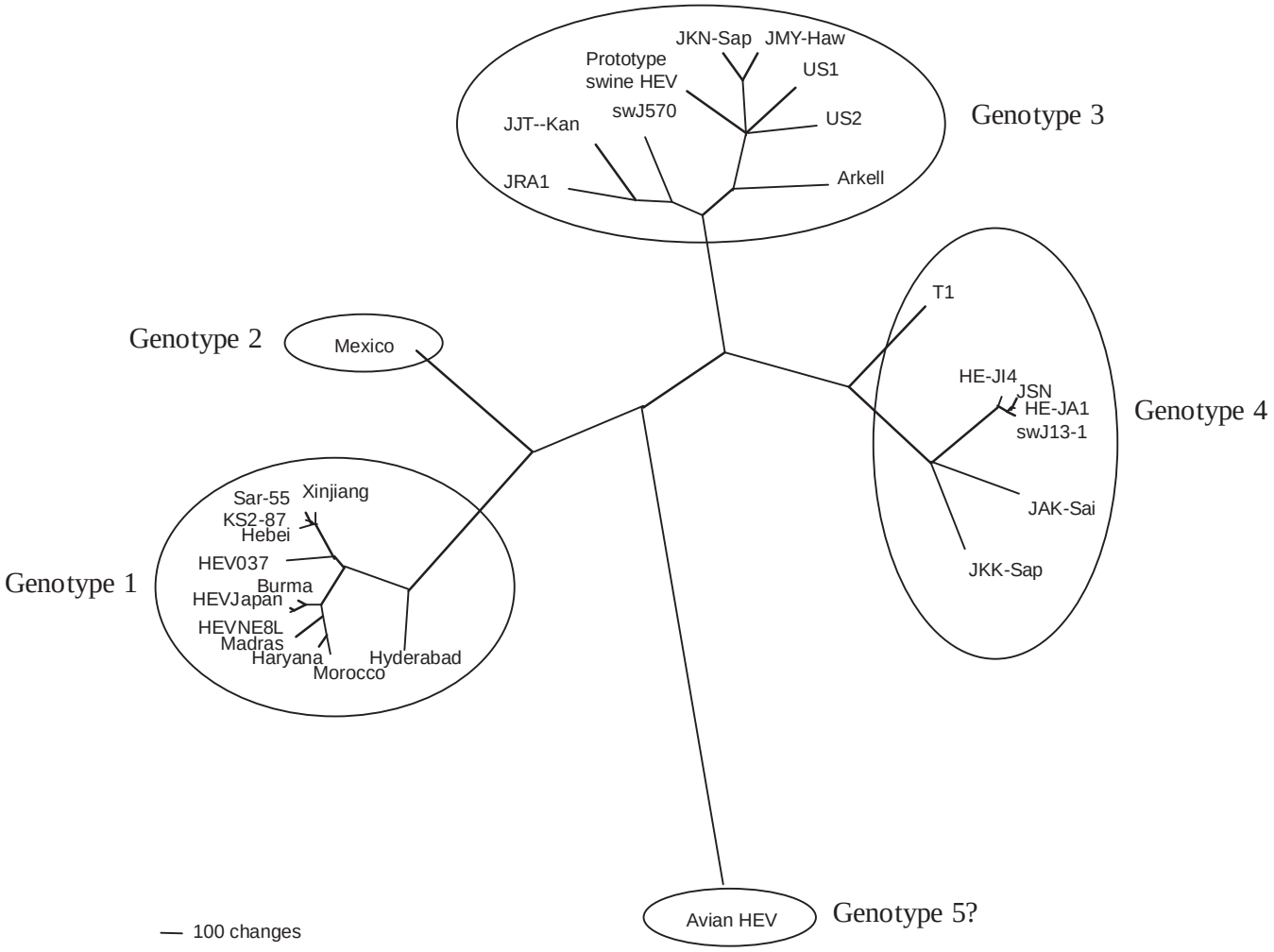
Hepatitis diseases caused by related hepatitis E viruses (HEV) (See Classification below) have been reported in humans (human HEV) and swine (swine HEV) (31, 32, 35, 37, 43). In addition, antibodies to HEV have been detected in numerous animal species including rodents, dogs, cats, sheep, goats, cattle and nonhuman primates, suggesting that these animal species have been infected by HEV of unknown classification (1, 33–36, 43). Unfortunately, the genotype of these infecting HEV and resulting seropositivity in many of these animal species, with the exception of pigs and chickens, could not be identified. Swine HEV from pigs can infect humans (33–37). However, human infections by avian HEV have not been reported.

Etiology

Bacteria could not be routinely isolated from affected livers (24, 45, 47, 49, 51, 55), except in one outbreak in which *Campylobacter spp.* were isolated (26). Attempts to link the cause of HS syndrome to toxins or bacterins were unsuccessful (44, 45, 55). It is now known that the primary causative agent of HS syndrome or BLS is a strain of hepatitis E virus (19, 40).

Classification

On the basis of its superficial similar genomic organization to caliciviruses, HEV was originally classified in the family Caliciviridae (43). However, as additional HEV sequences become available, it is clear that the genomic organization of HEV is quite different from that of the caliciviruses: the 5′ end of the HEV genome contains a cap structure that is absent in calicivirus, and HEV does not share significant sequence homology with caliciviruses (12, 28). Therefore, recently the International Committee on Taxonomy of Viruses officially declassified HEV from the Caliciviridae family and placed it in a new family Hepeviridae (13). All strains of HEV identified thus far, including avian HEV from chickens, belong to a single genus *Hepevirus*. At least 5 genotypes of HEV have been identified worldwide from humans and other animal species (22, 23): genotype 1 (Burmese-like Asian strains of human HEV), genotype 2 (a single Mexican strain of human HEV), genotype 3 (human HEV strains from sporadic cases in industrialized countries and swine HEV from pigs), genotype 4 (variant strains of human HEV from sporadic cases in Asia and swine HEV from pigs), and putative genotype 5 (avian HEV strains from the United States, Canada and Australia) (Figure 14.20).



14.20. A phylogenetic tree based on the complete genomic sequences of avian HEV (genotype 5) and 29 human and swine HEV strains (genotypes 1–4). The tree was constructed with the aid of the PAUP program by using heuristic search with 1,000 replicates. The scale bar, indicating the number of character state changes, is proportional to the genetic distance. Reproduced with permission from The Society for General Microbiology (22).

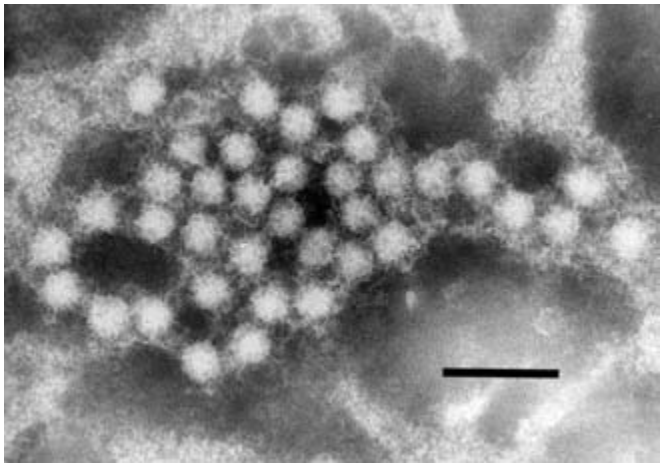
Morphology

Human HEV is a spherical, non-enveloped, symmetrical virus particle of approximately 32–34 nm in diameter with cup-shaped depressions on the surface, similar to caliciviruses (43). The avian HEV particles revealed by negative staining EM of bile samples from chickens with HS syndrome are similar in size and morphology to human HEV (Fig. 14.21).

Chemical Composition

The complete genome of avian HEV has been sequenced, and shown to be a polyadenylated, single-stranded positive sense RNA molecule of 6,654 bp in length excluding the poly (A) tail, which is approximately 600 bp shorter than that of human and swine HEVs. Similar to mammalian HEV genomes, the avian HEV genome consists of a short 5′ non-coding region (NCR) followed by three partially overlapping open reading frames (ORFs), and a 3′ NCR (Fig. 14.22). ORF1, located at the 5′ end of the genome, is believed to encode the nonstructural proteins. The predicted polyprotein encoded by ORF1 of avian HEV contained several putative functional domains including methyltransferase, papain-like cysteine protease, helicase, and RNA-dependent RNA polymerase (RdRp) that are also present in mammalian HEVs (Fig. 14.22), thus further supporting the conclusion that avian HEV is a member of the genus *Hepevirus*.

It has been shown that functional motifs typical of the helicase superfamily I and of the putative viral methyltransferase found throughout the alpha-like virus supergroup were conserved between avian HEV and mammalian HEVs (22, 28). ORF2 encodes the immunogenic capsid protein, and a truncated version of the avian HEV capsid protein has been expressed in bacteria and used for serological diagnosis of avian HEV infection (20, 21). ORF3 encodes a small protein with unknown function, although the ORF3 protein of human HEV has been shown to be a cytoskeleton-associated phosphoprotein that may be involved in virus replication (60). Complete genomic sequence analyses revealed that avian HEV shares approximately 50% nucleotide se-



14.21. Electron micrograph of negatively stained 30–35 nm diameter avian hepatitis E virus particles in bile sample from a chicken with hepatitis-splenomegaly syndrome. Bar = 100 nm. Reproduced with permission from The Society for General Microbiology (19).

quence identity in the complete genome with human and swine HEV strains (22). Phylogenetic analyses revealed that avian HEV represents a putative genotype 5 branch distinct from human and swine HEVs (22, 54) (Fig. 14.20).

Virus Replication

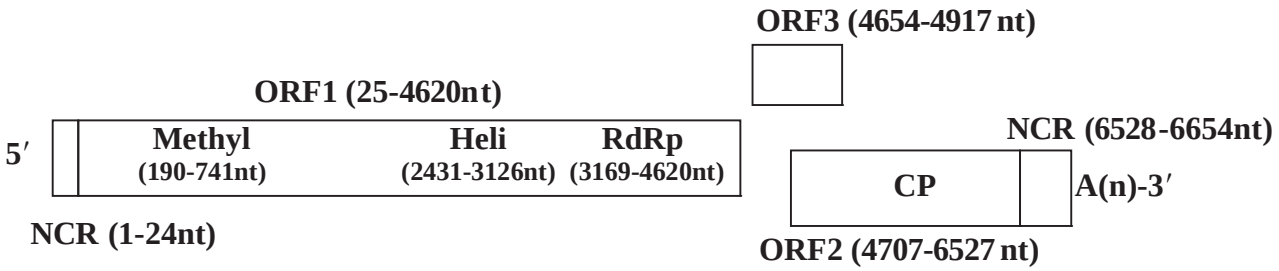
Due to the lack of a sufficient cell culture system for avian HEV or mammalian HEVs, the virus replication strategy is largely unknown. In specific-pathogen-free (SPF) chickens experimentally infected with avian HEV, replicating viruses were detected in livers as well as in several extrahepatic tissues including colon, cecum, jejunum, ileum, duodenum and cecal tonsils (5), indicating that avian HEV replicates not only in the liver but in the gastrointestinal tissues as well. The initial site of avian HEV replication in chickens is still unknown but it is believed that, prior to reaching its target organ liver, the virus first replicates in the gastrointestinal tract following oral ingestion of the virus (7, 59). Like swine and human HEVs, avian HEV is excreted in large amount in feces (4, 53).

Susceptibility to Chemical and Physical Agents

Little is known regarding avian HEV resistance to inactivation by physical, chemical or environmental forces. Most data known thus far are based on human HEV, but avian HEV and human HEV are likely similar in susceptibility to inactivation. Liver suspensions containing avian HEV remained infectious after treatment with chloroform and ether (11, 42) but lost infectivity after incubating at 56°C for 1 hour or 37°C for 6 hour. Avian HEV infectivity in liver suspensions was reduced 1000-fold after treatment with 0.05% Tween-20, 0.1% NP40, and 0.05% formalin (11, 41). Human HEV is sensitive to CsCl gradient centrifugation and low-temperature storage (6, 43). Iodinated disinfectants and autoclaving destroy the virus (3, 48). Human HEV virions are reportedly stable when exposed to trifluorotrichloroethane (56). Like other non-enveloped small RNA viruses, however, avian HEV can survive harsh environments. The fecal-oral route of transmission indicates that avian HEV is resistant to inactivation by acidic and mild alkaline conditions in the intestinal tract. It has recently been shown that human HEV is more heat labile than is hepatitis A virus (HAV), another enterically transmitted hepatitis virus (14). When fecal suspensions of a HAV or a human HEV strain were diluted in PBS buffer and compared in the same test by heating for one hour at 45, 50, 56, 60, 66, or 70°C, HAV was only 50% inactivated at 60°C but was almost totally inactivated at 66°C. In contrast, human HEV was about 50% inactivated at 56°C and almost totally inactivated (96%) at 60°C (14).

Strain Classification

The virus isolated from Australian chickens with BLS is a genetically variant strain of avian HEV (29–30, 40, 58) with approximately 80% nucleotide sequence identity with the avian HEV strains from the United States and Canada (2, 19–21, 54). Recently Sun et al (54) identified apparently avirulent strains of avian HEV from healthy chickens in normal chicken flocks in Virginia, and preliminary characterizations revealed unique genetic differences between the strains from chickens with HS syndrome and from healthy chickens (Billam and Meng, unpublished data). It remains to be studied whether or not the avian HEV strains recovered from healthy chickens in normal flocks are indeed non-pathogenic.



14.22. Schematic diagram of the genomic organization of avian HEV, which contains a short 5′ NCR, a 3′ NCR and three partially overlapping open reading frames (ORFs): ORF1 encodes non-structural proteins including putative functional domains of methyltransferase (Methyl), helicase (Heli) and RNA dependent RNA polymerase (RdRp); ORF2 encodes putative capsid protein (CP), and ORF3 encodes a small protein with unknown function. The beginning and ending nucleotide (nt) positions of NCRs and ORFs are indicated in parentheses. Reproduced with permission from The Society for General Microbiology (22).

Avian HEV is not only genetically, but also antigenically, related to swine and human HEVs (15, 16, 19, 20, 22). It has been demonstrated that the avian HEV capsid protein reacted with antisera against a genotype 1 human HEV, and genotype 3 human and swine HEVs. Convalescent sera from chickens experimentally infected with avian HEV also reacted with recombinant capsid proteins of genotype 3 swine HEV and genotype 1 human HEV (20).

Laboratory Host Systems

HEV is notoriously difficult to propagate *in vitro.* Avian HEV can be propagated in chicken embryos only when the virus is inoculated intravenously but not by other conventional inoculation methods (8, 38, Haqshenas and Meng, unpublished data). It has been demonstrated that LMH chicken liver cells (ATCC CRL-2117), when transfected with RNA transcripts from infectious cDNA clones of avian HEV, supported avian HEV replication (23). Viral antigens were detected in transfected LMH cells by immunofluorescence assay with avian HEV antiserum, and the fluorescent signals were mainly in the cytoplasm. Approximately 10–15% of the cells were positive for avian HEV antigens, however the virus does not spread from cell to cell (23).

Pathobiology and Epidemiology

Incidence and Distribution

First reported in western Canada in 1991 (47), HS syndrome has now been recognized in eastern Canada (2, 55), California (45), and the midwestern and eastern United States (26, Shivaprasad, unpublished data). BLS has been reported in Australia (8, 9, 10, 18, 38, 40), and serological evidence of avian HEV infection was also reported in the United Kingdom (57). Leghorn hens in cages are typically affected and HS syndrome frequently reoccurs on some farms (45, 47). The disease has also been recognized in broiler breeder hens (24, 49, 50), and may be associated with sporadic mortality in dual-purpose hens and in small flocks kept on litter (25).

In the United States, avian HEV infection is enzootic in chicken flocks. A recent serological survey for the prevalence of avian HEV antibodies included a total of 1,276 chickens of different ages and breeds from 76 different flocks in five states (CA, CO, CT, VA, and WI) (21). It was found that approximately 71% chicken flocks and 30% chickens in the United States were positive for antibodies to avian HEV (21). Approximately 17% of young chickens (less than 18 weeks of age) and about 36% of adult chickens were positive for avian HEV-specific antibodies.

Natural and Experimental Hosts

Under field conditions, chickens are the only known host for avian HEV infections. Under experimental conditions, chickens of all ages are susceptible to avian HEV infection (4, 5, 23, 38, 53). SPF chickens were experimentally infected with avian HEV via both intravenous and oronasal routes of inoculation (4). Embryonic chicken eggs are also susceptible to infections by avian HEV but only via intravenous route of inoculation (38, Haqshenas and Meng, unpublished data). Under laboratory conditions, eight-week-old turkeys intravenously inoculated with an infectious stock of avian HEV also became infected as evidenced by seroconversion to avian HEV antibodies, viremia, and fecal virus shedding (53). However, attempts to experimentally infect rhesus monkeys (22) and mice (Sun and Meng, unpublished data) with avian HEV were unsuccessful.

Transmission, Carriers, Vectors

Transmission within and between flocks appears to occur readily. In a prospective study of natural avian HEV infection in a chicken flock (54), all 14 chickens monitored in the study were seronegative at 12 weeks of age. The first chicken became seroconverted at 13 weeks of age, and by 21 weeks of age all 14 chickens in the flock had seroconverted (54). Like human and swine HEVs, the transmission route for avian HEV is presumably fecal-oral, and experimental avian HEV infection has been successfully reproduced via oronasal route inoculation of SPF chickens (4). Feces from infected chickens are likely the main source of virus for transmission as large amounts of virus are shed in feces in experimentally infected chickens (4, 53). Other routes of transmission cannot be ruled out at this time. Vertical transmission has been suggested during avian HEV infection of hens (9), and experimental aerosol transmission of avian HEV was unsuccessful (10, 11). It has been demonstrated that uninoculated chickens housed in the same room with avian HEV-inoculated chickens became infected through direct contacts with infected ones (53). There is no known carrier or vector implicated in the transmission of avian HEV, although rodents on the chicken farms might serve as a mechanical carrier (Sun and Meng, unpublished data).

Clinical Signs

The incubation period, from the time of infection to virus shedding in feces, ranged from 1 to 3 weeks in oronasally-infected chickens (4). The disease (HS syndrome or BLS) morbidity and mortality in the field are relatively low, although subclinical avian HEV infections are widespread in chicken flocks in the United States and perhaps in other countries as well (21, 54). No clinical sign has been recognized in birds with HS syndrome prior to death (45, 47, 49, 55). In some outbreaks, there has been a drop in egg production of up to 20% (45, 47), but in other outbreaks egg production has not been affected (55). HS syndrome is characterized by above-normal mortality in broiler breeder hens and laying hens of 30–72 week of age, with the highest incidence occurring between 40–50 weeks of age (46, 49). Weekly mortality increases to approximately 0.3% for several weeks during the middle of the production period and may sometimes exceed 1.0% (45, 47, 49, 55). The clinical signs for BLS in Australia also vary from subclinical infection to egg drops that may reach 20% and accompanied by up to 1% mortality per week over a period of 3–4 weeks (9, 18). Diseased birds may have pale combs and wattles, depression, anorexia, and soiled vent feathers or pasty droppings (9, 10, 18, 41, 46). Small eggs with thin and poorly pigmented shells are produced in affected flocks, however the internal quality, fertility and hatchability of the eggs are unaffected (41, 42). Affected flocks in the United States and Europe

appear to have milder or subclinical infections compared to those in Australia (42, 54).

Pathology

Under field conditions, dead chickens usually have regressive ovaries, red fluid in the abdomen, and enlarged liver and spleen (18,46, 47, 49, 55). Prior to death, affected birds are usually in good condition, with pale combs and wattles (55), but sometimes birds in poor condition are also seen (47). Livers are enlarged with hemorrhage and/or clotted blood can be seen in the abdominal cavity (Fig. 14.23).

Livers can often be friable, mottled and stippled with red, yellow, and/or tan foci, and may have subcapsular hematomas and attached blood clots on the surface (45, 49). Presence of clotted blood in the abdominal cavity and hemorrhages in the liver can be confused with hemorrhagic fatty liver syndrome (HFLS), which is a common condition in laying type chickens, but the livers are not fatty in HS syndrome. Spleens from affected birds are mild to severely enlarged (Fig. 14.24), sometimes with white mottling (24, 45,49, 55). Affected birds generally have regressive ovaries (45, 47), but some also have active ovaries (55).

Microscopically, liver lesions vary from multifocal hemorrhage to extensive areas of necrosis and hemorrhage and infiltration of heterophils and mononuclear inflammatory cells around portal triads. There is often segmental infiltration of lymphocytes and a few plasma cells in and around portal veins. Also, accumulation of homogenous eosinophilic material, amyloid in the interstitium of the liver and separation of hepatocytes are common. In severe cases, discrete granulomas and possible thrombosis of portal veins were recognized. Lesions in spleens consist of lymphoid depletion accompanied by an increase in the cells of the mononuclear phagocyte system in later stages. There is accumulation of homogenous eosinophilic material, amyloid in the walls of small arteries and arterioles and in the interstitium. Eosinophilic material in both livers and spleens was identified as amyloid using Congo red stain (45, 49, 50, 51, 55) (Fig. 14.25).

Under experimental conditions, gross lesions were observed primarily in the liver of SPF chickens experimentally infected with avian HEV (4). Subcapsular hemorrhages, and a slightly enlarged right intermediate lobe of the liver (Fig. 14.26) were observed in approximately one-fourth of the infected chickens (4).

Microscopically, lymphocytic periphlebitis and phlebitis foci were observed in liver sections (Fig. 14.27). The severity of liver lesions peaked at 10 days post-inoculation (DPI) in the intravenously inoculated chickens. Other liver lesions such as foci of hepatocellular necrosis, amyloid in the interstitium, and subcapsular hemorrhages were also observed in some chickens (Fig. 14.27). Microscopic lesions were also observed in spleen (mild lymphoid hyperplasia), thymus (mild cortical hypoplasia), kidney (occasional mild lymphocytic interstitial nephritis), and lung (mild lymphocytic and heterophilic parabronchial and interstitial inflammation) of SPF chickens infected with avian HEV (4). Microscopic lesions were generally absent in the tissues collected from gastroenteric tract. There was no significant elevation of serum levels of liver enzymes AST, albumin/globulin (A/G) ratios or bile acids (4). However, LDH levels behaved differently over time ($P = 0.0851$). In intravenously-inoculated chickens, LDH levels peaked at 1 week post-inoculation (wpi) and then returned to baseline levels. The LDH levels in oronasally-inoculated chickens remained elevated from 1 to 4, and 6 wpi prior to returning to baseline values at 7 wpi. The oronasally-inoculated chickens having higher total proteins than intravenously-inoculated and control group chickens ($P<0.0001$) (4).

Pathogenesis of the Infectious Process

The pathogenesis of avian HEV infection in chickens is largely unknown. It is believed that, like mammalian HEVs (43, 59), avian HEV enters the host through the fecal-oral route. However, the primary site of avian HEV replication in chickens is not yet known. In primates and pigs experimentally infected with human HEV or swine HEV, virus replication in the liver has been demonstrated (17, 32). It is believed that, after replication in the liver, HEV is released to the gallbladder from hepatocytes and then is excreted in feces. Williams et al (59) showed that there exist extrahepatic sites of HEV replication in pigs experimentally infected with swine HEV and human HEV. To identify the extrahepatic sites of avian HEV replication in chickens, Billam *et al.* (5) employed a negative-strand-specific RT-PCR that can detect replicating avian HEV RNA in tissues. It was found that, in addition to the liver, replicating avian HEV was also detected in colon tissues at 5, 16, 20 and 35 dpi, in cecum and jejunum tissues at 20 and 35 dpi, in ileum tissues at 7, 10, 20 and 35 dpi, in duodenum tissues at 20 dpi, and in cecal tonsils at 35 and 56 dpi of experimentally infected chickens (5). Therefore, the gastrointestinal tissues appear to be the first site of avian HEV replication following oral inoculation. However, the clinical and pathological significances of these extrahepatic sites of HEV replication remain unknown.

Immunity

The humoral immune response in chickens infected with avian HEV, characterized by the appearance of IgG antibodies, appears at approximately 1 to 4 weeks post-inoculation (4, 53). The cell-mediated immunity in response to avian HEV infection in chickens is unknown. Like mammalian HEVs, the capsid protein of avian HEV is immunogenic and induces protective immunity against avian HEV infection (16). It has been demonstrated that the avian HEV capsid protein shares common antigenic epitopes and cross-reacts with that of swine and human HEVs (15, 16, 20). Avian HEV capsid protein reacted with antisera against human HEV (Sar-55 strain) and with convalescent antisera against swine HEV and the US2 strain of human HEV. Conversely, convalescent sera from chickens experimentally infected with avian HEV also reacted with the recombinant capsid proteins of swine and human HEVs (20). A total of 4 putative antigenic domains (I, II, III, IV) have been identified in the avian HEV capsid protein. Recently Guo *et al.* (15) identified a B-cell epitope at the C-terminus of domain II (possibly between aa 477–492) that is unique for avian HEV, a B-cell epitope in domain I (aa 389–410) that is common to avian, human and swine HEVs, and one or more B-cell epitopes in domain IV (aa

583–600) that are shared between avian and human HEVs. It appears that all HEV strains identified thus far belong to a single serotype.

Diagnosis

A presumptive diagnosis of HS syndrome can be made on the basis of clinical signs and gross and microscopic lesions. However, HS syndrome needs to be differentiated from hemorrhagic fatty liver syndrome (HFLS) due to the presence of clotted blood in the abdominal cavity and hemorrhages in the liver with HS syndrome. The livers in HS syndrome are not fatty as in HFLS. Clotted or unclotted blood in the abdominal cavity or around the liver sometime can also be seen in cases of rodenticide (anticoagulants) toxicities. Trauma to the body or the liver with blood in and around liver in a chicken should also be considered for differential diagnosis. Virus particles of 30–35 nm may be detected in bile of chickens with HS syndrome by negative staining electron microscopy.

Avian HEV does not replicate in cell culture. Although embryonic chicken eggs can be experimentally infected with avian HEV via intravenous inoculation (38, Haqshenas and Meng, unpublished data), virus isolation with chicken embryos is not practical due to the technical difficulty and high mortality associated with the intravenous inoculation procedure. Currently, the diagnosis of avian HEV infection is primarily based on detection of the virus RNA by RT-PCR or detection of antibodies by ELISA (21, 53, 54). However, the sensitivity and specificity of these assays are largely not known. A truncated version of the avian HEV capsid protein has been expressed and used in an ELISA to detect avian HEV antibodies in chickens (4, 20–21, 53–54). An agarose gel immunodiffusion test (AGID) and an ELISA assay using purified antigen extracted from the spleen and liver of affected chickens have been developed to detect avian HEV infection in Australia (11, 40, 57).

Serological assays alone are inadequate in screening for acute avian HEV infection. Viremia and fecal virus shedding occur in infected birds much earlier than the appearance of avian HEV IgG antibodies and thus, seronegative birds could still be infected with avian HEV (4, 53–54). Avian HEV-specific RT-PCR assays have been successfully developed for the detection of avian HEV infections in chickens (4, 21, 27, 53, 54). However, the specificity of the RT-PCR assays in detecting avian HEV strains in chickens from different geographic regions is not known, since avian HEV strains identified from chickens in different geographic regions are genetically heterogenic (21, 54). Therefore, genetic identification and characterization of additional field strains of avian HEV from chickens in different geographic regions will be critical for developing a universal RT-PCR assay that can detect all strains of avian HEV. A heteroduplex mobility assay has recently been developed as a pre-sequencing tool to identify genetically divergent strains of avian HEV (52). The recent identification of antigenic epitopes unique to avian HEV capsid protein (15) will allow future development of differential diagnostic assays to distinguish infections caused by avian HEV, swine HEV or human HEV.

Intervention Strategies

A vaccine against avian HEV or mammalian HEVs is not yet available. Currently there is no treatment for avian HEV infection. Implementation of strict biosecurity in chicken farms may limit the spread of virus.

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Chapter 15

Neoplastic Diseases

Introduction

Aly. M. Fadly

Neoplastic diseases of poultry comprise a variety of related and unrelated conditions possessing a single common denominator: neoplastic character. This group of diseases is divided into two main categories, depending on whether the etiologic agent is known. In addition to causing economic loss from tumor mortality as well as poor performance, some of these neoplastic diseases have served as highly suitable models for studying various phenomena of neoplasia. Indeed, medical research has found avian oncology an abundant resource (4). This chapter deals primarily with the three most economically important virus-induced neoplastic diseases of poultry, namely Marek's disease, caused by a herpesvirus, and the avian leukoses and reticuloendotheliosis, caused by retroviruses. Virus-induced tumors are principally of mesodermal origin and are transmissible. These neoplastic diseases or disease complexes are described, each in a separate section because of its etiologic distinctness.

The first section describes Marek's disease (MD), a T-cell lymphoma primarily of chickens caused by a highly cell-associated alphaherpesvirus, although the lymphotropic properties of MD virus are similar to those of gammaherpesviruses. MD lymphoproliferative lesions involve the peripheral nervous system as well as other tissues and visceral organs. MD is and has been controlled since early 1970s by use of conventional vaccines. During the last three decades, research on MD has resulted not only in improved conventional vaccines, but also in improved methods of vaccination, and a better understanding of host genetic resistance to the disease. However, despite widespread use of vaccines and development of new methods of vaccination, economic losses from mortality of layers and breeders and condemnation of broilers continue to occur (12, 18, 23). Further, MD has been diagnosed in commercial turkey flocks in France, Germany, Israel, and Ukraine (6, 7, 15, 16, 23), suggesting that the host range of MDV has apparently expanded to include turkeys. Clearly, in the absence of control measures, MD is capable of causing devastating losses in commercial layer and broiler flocks (24, 25). As a disease occurring worldwide, with reports of vaccination breaks and probable emergence of more virulent pathotypes, MD poses severe threats to the poultry industry and developing strategies for its control remains one of the great challenges today (18, 23, 24, 25).

A second section describes a group of leukoses, sarcomas, and related neoplasms induced by a number of closely related group of avian retroviruses termed the leukosis/sarcoma (L/S) viruses. The term *leukosis* is used because a leukemic blood picture is not always present during the course of leukemia-like proliferative diseases of the hemopoietic system (11, 18). The various forms of hemopoietic system neoplastic changes induced by the L/S group of avian retroviruses include the lymphopoietic (lymphocytic) system, the erythropoietic (red cell) system, and the myelopoietic (myelocytic) system. Lymphoid leukosis, a lymphoproliferative disease of chickens affecting primarily the bursa of Fabricius and visceral organs, is the most common form of leukosis that arise from infection with a member of the L/S group of viruses known as avian leukosis virus (ALV) (5, 8, 11, 18). Other neoplasms of hematopoietic origin that can also be seen in ALV-infected chickens, albeit infrequently, include erythroblastosis, myeloblastosis, myelocytomatosis, and certain related neoplasms such as nephroblastoma and osteopetrosis. However, with the recognition of subgroup J ALV infection, myelocytomatosis, as a neoplastic condition was frequently diagnosed during the 1990s, particularly in affected breeders of meat-type chickens (17, 18). These conditions, along with sarcomas and other connective tissue tumors, are etiologically related and are discussed as a group.

The third section describes reticuloendotheliosis (RE), a group of disease syndromes caused by an avian retrovirus unrelated to the L/S group of viruses termed RE virus (REV) (20, 21, 26). The most common clinical diseases induced by REV are chronic lymphomas and an immunosuppressive runting disease. Although REV infrequently causes clinical disease, the virus is widespread. REV infects chickens, turkeys, ducks, geese, pheasants, quail, and probably many other avian species. However, the principal economic concerns of REV infection are as contaminants of biologic products produced in chicken embryo cells or tissues or as a barrier to export of breeding stock to certain countries (9, 24, 26).

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Table 15.1. Transmissible neoplasms.

Virus type	Nucleic acid type	Virus classification of etiological agent	Neoplastic diseases
Retrovirus	RNA	Leukosis/sarcoma group	Leukoses
			Lymphoid leukosis
			Erythroblastosis
			Myeloloblastosis
			Sarcomas and other connective tissue tumors
			Fibrosarcoma, fibroma
			Myxosarcoma, myxoma
			Osteogenic sarcoma, osteoma
			Histiocytic sarcoma
			Related neoplasms
			Hemangioma
			Nephroblastoma
			Hepatocarcinoma
			Osteopetrosis
		Reticuloendotheliosis group	Reticuloendotheliosis
			Lymphoid leukosis
Herpesvirus	DNA	Marek's disease virus	Marek's disease

The fourth section describes tumors of unknown etiology on the basis of morphologic characteristics. Included are a wide variety of benign and malignant neoplasms derived from muscle, epithelial, and nerve tissues; serous membranes; and pigmented cells.

A neoplastic disease of turkeys known as lymphoproliferative disease (LPD) that had been reported in Europe and Israel is induced by yet another retrovirus distinct from both the L/S and RE viruses (2). The incidence of LPD of turkeys has always been sporadic (10, 24); therefore discussion of this rare neoplastic syndrome of turkeys is not included in this chapter.

Because many of the avian tumor viruses appear to have multipotent characteristics, i.e., they can sometimes induce a variety of neoplasms, classification and nomenclature of these virus-induced neoplasms present a problem. The dilemma is largely due to the fact that certain strains of these viruses induce some pathologic lesions difficult to distinguish from those induced by another unrelated virus. The two prevalent lymphomatotic diseases, namely MD and lymphoid leukosis, are particularly confusing. Although REV-induced lymphoid tumors are observed only infrequently such as in cases of using contaminated vaccines, or only under experimental conditions, they may also add to the confusion. The problem is compounded by the fact that avian tumor viruses are widespread and infection in the absence of tumor formation is common. Recently, Witter *et al.* (27) introduced a practical and useful strategy for the differential diagnosis of viral lymphomas in chickens.

Choice of terminology for this chapter (Table 15.1) is based on that originally adopted by the World Veterinary Poultry Association (3) and includes modifications in current use. The classification system accompanying this nomenclature is especially suited to the mode of presentation that follows, i.e., categorization of diseases or disease complexes by agent type instead of pathologic manifestation. Subdivision within agent-type diseases by pathologic expression has been employed where it seemed appropriate.

Incidence and importance of neoplasms in poultry can only be generally estimated. Feldman and Olson (13) quoted reports (1915–55) in which the incidence of tumors, except neurolymphomatosis and osteopetrosis, varied from 3 to 19%. More recently, we have had the advantage of data accumulated by the United States Department of Agriculture (USDA) from federally inspected slaughtered poultry. These data showed that incidence of "leukosis" in young chickens (probably nearly all MD) increased dramatically during a 10-yr period beginning in 1961.

There was a gradual rise in leukosis condemnations in young chickens from about 0.1% in 1961 to over 1.5% in 1968–70 (see (19)). After 1970, the trend was reversed and condemnations of young birds returned to 1961 levels, undoubtedly the result of MD vaccination of broilers. During peak years, however, over 40 million young birds (nearly 50% of all condemnations) were condemned for leukosis, and it was conceded that it was one of the most serious problems confronting the poultry industry.

Condemnations due to leukosis in mature birds were fairly consistent and much lower (less than 0.5 million birds, usually less than 10% of all condemnations). Condemnation rates with other tumors, many of which were leiomyomas of the mesosalpinx, were actually much higher (up to five times) than those from leukosis. In some cases, condemnations due to squamous cell carcinoma exceed that of leukosis and can have at least some economic impact (see subchapter on squamous cell carcinoma). Because most losses from leukotic diseases occur during growing and productive periods of layers, presence of gross lesions at slaughter is a poor index of their true incidence and importance.

Annual losses in the United States were placed at more than $150 million in 1967, prior to the introduction of MD vaccines (1). In 1985, Purchase (19) estimated that benefits derived by the poultry industry in the United States as a result of MD vaccine totaled nearly $170 million annually. This included increased egg production and decreased losses from non-MD causes as indirect benefits as well as the direct effect of lowered MD mortality and condemnations. Lymphoid leukosis losses may be significant in some flocks, but mortality probably constitutes only a small portion of the economic loss from that disease. Studies by Gavora *et al*. (14) and Spencer *et al.* (22) have shown that lowered egg production and increased mortality from causes other than lymphoid leukosis are associated with infection by lymphoid leukosis virus, and the total economic impact from these could be extremely significant. However, present and past efforts by the primary breeders to reduce the infection rate or to eradicate infection have markedly lower this impact (24). Generally, the incidence of neoplasms other than lymphoid tumors appears to be low and of questionable economic significance.

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Marek's Disease

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Introduction

Marek's disease (MD) is a common lymphoproliferative disease of chickens, usually characterized by mononuclear cellular infiltrates in peripheral nerves and various other organs and tissues including iris and skin. The disease is caused by a herpesvirus, is transmissible, and can be distinguished etiologically from other lymphoid neoplasms of birds.

Because the literature on MD has greatly expanded, it is no longer feasible to cite all relevant publications that provide the scientific basis for our current knowledge of the disease. In this chapter, literature is cited selectively, and reviews are often substituted for original papers. Readers are encouraged to look elsewhere for additional details. Useful sources of information on MD are the books *Marek's Disease: Scientific Basis and Methods of Control*, *Marek's Disease*, and *Marek's Disease. An Evolving Problem* edited by respectively, L. N. Payne (481), K. Hirai (263),and F. Davison and V. Nair (183) and the proceedings from 7 international symposia on MD conducted in 1978, 1984, 1988, 1992, 1996, 2000, and 2004.

Definition and Synonyms

The old terminology was confusing because early authors used a variety of names to designate the lymphoproliferative and neurological aspects of the disease. Also, the differentiation between inflammatory and neoplastic aspects originally were confused, probably because the virus strains were less virulent and induced few if any lymphomas. The seminal description by József Marek (408) identified the disease as *polyneuritis*. Other common synonyms included *neuritis*, *neurolymphomatosis gallinarum*, and *range paralysis*. Jungherr and colleagues (323) proposed that the term *lymphomatosis* be subdivided into *visceral*, *neural*, and *ocular* forms. This nomenclature was widely applied for more than 20 years to all lymphoproliferative diseases (also called *leukosis*) of chickens, including MD, but masked the etiological distinctions between MD and lymphoid leukosis (LL). In retrospect, neural and ocular lymphomatosis were probably MD; whereas visceral lymphomatosis included both MD and LL. The term *acute leukosis* or *acute Marek's disease* was used in the 1960s to designate more virulent manifestations of MD characterized by visceral lymphomas. Lymphomatous lesions in the skin, which were common in broilers, were sometimes termed *skin leukosis*. In 1961, Biggs (53, 59) proposed the term *Marek's disease* to distinguish the condition clearly from etiologically different lymphoproliferative diseases. This term is in common use.

The authors are greatly indebted to Bruce W. Calnek and Richard L. Witter for their contributions to earlier editions of this chapter.

Drs. Bruce W. Calnek and Dr Richard L. Witter were the co-authors of 5 and 6, respectively, prior editions of this chapter, encompassing a 25- to 30-year span. Their inspiration, along with many of their words, is reflected in the current chapter.

MD has also been subdivided into *acute* and *classical* forms, where the latter term designates forms of the disease prevalent prior to the 1950s (54). MD virus can also induce other clinically distinct disease syndromes such as *transient paralysis*, *early mortality syndrome*, *cytolytic infection*, *atherosclerosis*, and *persistent neurological disease.*

Economic Significance

Prior to the use of vaccines, MD constituted a serious economic threat to the poultry industry causing up to 60% mortality in layer flocks and 10% condemnations in broiler flocks. Because vaccines are not 100% effective, sporadic losses still occur, but they are no longer as serious a problem. Purchase (526) estimated that mortality and condemnation losses due to MD totaled about $12 million in the United States in 1984. When combined with economic loss from the costs of vaccine and application and reduced egg production, however, the total was about $169 million in the United States and $943 million worldwide. Morrow and Fehler (432) quoted current worldwide, annual losses in the range of US$ 1 to 2 billion, but they indicated that these figures are impossible to verify. The disease remains a major concern for the poultry industry due to the unpredictability of outbreaks and the possibility that vaccines may ultimately fail as a consequence of the evolution of more virulent strains of MD virus (MDV).

Public Health Significance

Purchase and Witter (530) have reviewed the literature related to MD and human health concerns, particularly human cancer. They cite numerous reports of virologic, pathologic, serologic, and epidemiologic studies that support a conclusion that no etiologic relationship exists between MDV or any of the MD vaccine viruses and human cancer. The public health significance of MD has attracted little attention. A role for MDV in the etiology of multiple sclerosis of humans has been suggested (404, 405, 415) but was later refuted (257). Recently, a similar claim was made for HVT or HVT-like viruses (71) lacking any supportive evidence for this speculation. The presence of DNA sequences of the gD gene of MDV was described in sera from humans with and without exposure to poultry (363, 364), but genes unique to MDV were not analyzed in these studies. However, Hennig *et al.* (256) were unable to detect any MDV sequences in 300 human plasma samples by quantitative real-time PCR. At this time, no compelling evidence suggests that MDV infects humans or adversely affects human health.

Scientific Significance

MD has provided a fertile environment for contributions to veterinary medicine, basic science, and comparative oncology. The disease itself is uncommonly complex, featuring an interplay of neoplasia and inflammation expressed as several distinct clinical syndromes, each modified in important ways by host genetic in-

fluences. MDV, an alphaherpesvirus with lymphotropic properties of gammaherpesviruses, is highly cell-associated but readily transmitted, and its virulence varies and evolves. It has 2 unique sister viruses, both nononcogenic, that naturally infect chickens and turkeys. Infection induces complex immune responses usually resulting in high levels of protection. Vaccination for MD constitutes an outstanding example of successful disease control in veterinary medicine. Moreover, MD vaccines are the first effective vaccines against cancer in any species.

History

The seminal report by József Marek, published in 1907 (408), of paresis in 4 roosters is the first account of the disease that now bears this author's name. Outbreaks were reported as early as 1914 in the United States, and subsequently, the disease was recognized in The Netherlands, Great Britain, and many other countries. The detailed description by Pappenheimer *et al.* (471) clarified the lymphoproliferative nature of the lesions in peripheral nerves and spinal ganglia and established, as part of the syndrome, lymphoid neoplasms in the ovary and other visceral organs.

Many early attempts at transmission were equivocal or unsuccessful due to the lack of susceptible chickens and adequate biocontainment and to the failure to recognize the cell-associated nature of the causative agent. However, experiments initiated by Hutt and Cole in 1935 established the role of genetic resistance in the disease and ultimately provided susceptible chicken lines for transmission studies (282, 283).

The disease gradually increased in severity. Pappenheimer *et al.* (471) reported mortality from fowl paralysis reaching 20% in one pullet flock as early as 1922. A marked increase in mortality in commercial chickens occurred between 1925 and 1937, at least half of which was due to fowl paralysis and other neoplasms (685). Industry concerns prompted the appropriation of federal funds to construct a new laboratory in East Lansing, Michigan, dedicated to this disease. Its initial projects, launched in 1939, were directed toward the genetics and pathology of fowl paralysis in chickens.

An acute form of the disease in broiler chickens, characterized by tumors in multiple viscera, muscle, and skin, was first recognized in the mid-1950s (50). By the early 1960s, it was a major cause of condemnation of broiler carcasses (51). The incidence continued to increase during the 1960s, and by 1970 about 1.5% of all broilers in the United States were discarded at processing with neoplastic lesions. Mortality in pullets and layers reached as high as 30–60%, and the poultry industry was facing an economic crisis. The definitive transmission of MD, established in the early 1960s (64, 593), confirmed the infectious nature of the disease and provided an experimental system for its study. The avid cell association of the agent (65) made identification of the agent elusive. Isolation of a herpesvirus in cell cultures inoculated with cells from diseased chickens was reported in 1967 by 2 independent laboratories (154, 443, 631). This virus was confirmed as the etiological agent through association studies (60, 706) and, ultimately, by transmission with cell-free virus obtained from feather follicles (110). The virus became attenuated upon serial passage in chicken kidney cultures (156), and the attenuated virus, when inoculated into newly hatched chicks, provided protection against subsequent challenge with virulent strains (157). Naturally avirulent, antigenically related strains were found in turkeys (718) and chickens (63, 143) and provided protective immunity. Thus, in the span of a very few highly productive years, the causative agent was identified, and vaccination was established as an effective control strategy.

Meanwhile, Calnek *et al.* (110, 114, 130) found that the virus was shed in a cell-free, fully infectious form from the feather follicle providing an explanation for the highly contagious nature of the disease. The virus was relatively stable in the poultry house environment, and respiratory exposure to dust or dander appeared to be the mechanism of natural transmission (48).

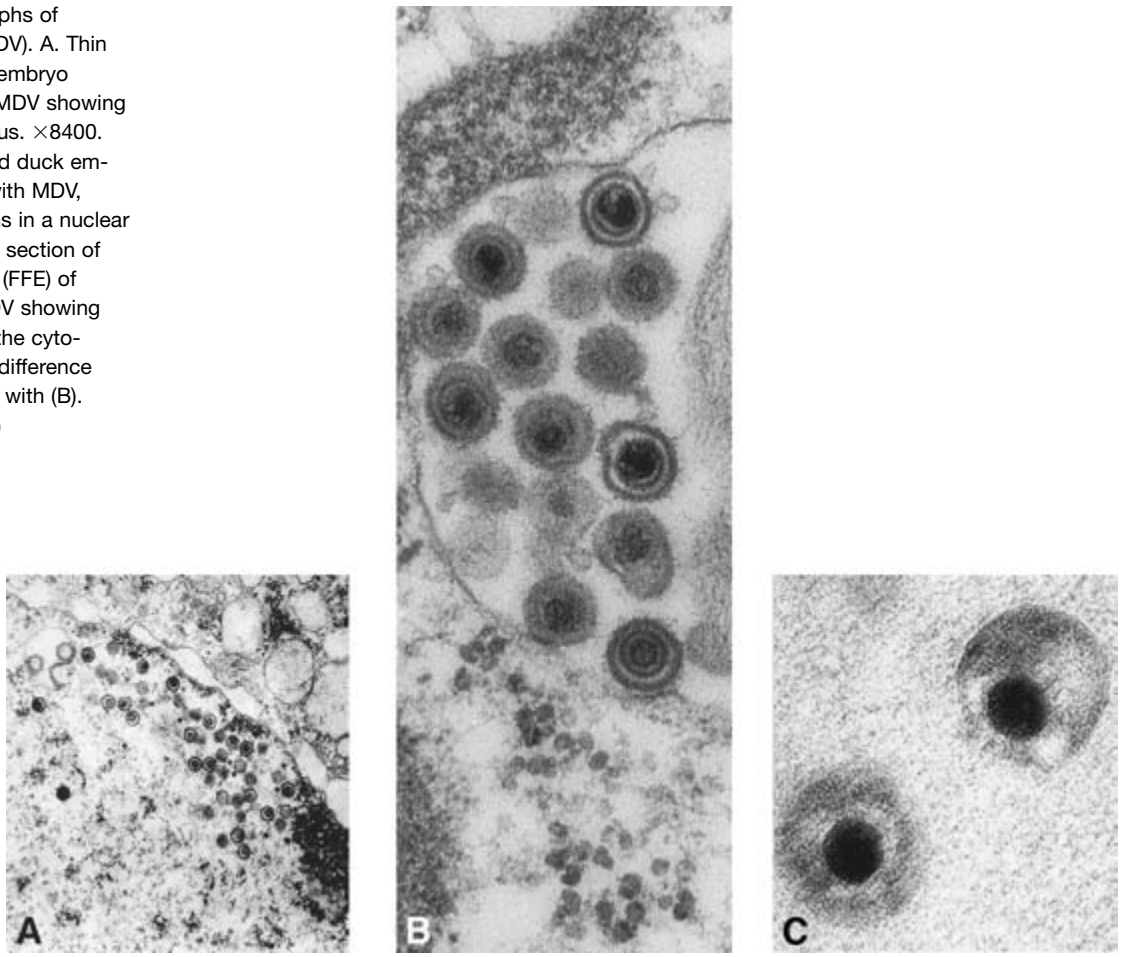
The ensuing years continued the stream of new findings about MD. Important contributions prior to 1985 include the development of lymphoblastoid cell lines from MD tumors (13) characterization of the T-cell nature of the tumor cell (280) identification of cytolytic and immunosuppressive phases of the disease (485), linkage of transient paralysis with MDV infection (343), isolation of the CVI988 (546) and SB-1 (571) strains, which proved to be successful vaccines, and identification of 3 viral serotypes based on antigenic characteristics (370, 676). Genetic resistance was linked to the B-locus or major histocompatibility complex (MHC) (77, 249). The stages of infection from respiratory tract to cytolytic infection of B cells, to activation, latent infection, and ultimately transformation of T cells were elucidated (105, 127, 613). Synergism among vaccine viruses was identified (574, 688) and contributed to the development of bivalent vaccines (123, 722).

These and other important findings that laid the basis for our current understanding of the disease are detailed in several historical reviews (e.g., 58, 59). Advancements of knowledge on MD during the 1960s, leading to effective vaccines, were showcased during the 5th International Symposium on Marek's disease in 1996, and relevant information is available in the proceedings, the videotape "Legacy of the 1960s," and the historical archives of the American Association of Avian Pathologists.

Etiology

Classification

MDV is a cell-associated herpesvirus (citations in 564) with lymphotropic properties similar to those of gammaherpesviruses. However, its molecular structure and genomic organization are similar to alphaherpesviruses (83, 375, 664). McGeoch *et al.* (414) proposed to place all MDV serotypes in a separate subgroup, a3, of the alphaherpesvirinae. As per the recent classsification by the International Committee on Taxonomy of Viruses (ICTV) (http://www.ncbi.nlm.gov/ICTVdb/ICTVdB), all MDV serotypes are grouped together in the genus *Mardivirus* (284) within the subfamily *Alphaherpesvirinae*. Members of the genus *Mardivirus*, described as belonging to the 3 serotypes (see below) are now grouped as the 3 species *viz. Gallid herpesvirus* 2 (serotype 1), *Gallid herpesvirus* 3 (serotype 2) and *Meleagrid*



15.1. Electron micrographs of Marek's disease virus (MDV). A. Thin section of cultured duck embryo fibroblasts infected with MDV showing scattered virions in nucleus. ×8400. B. Thin section of cultured duck embryo fibroblast infected with MDV, showing enveloped virions in a nuclear vesicle. ×60,000. C. Thin section of feather follicle epithelium (FFE) of chicken infected with MDV showing enveloped virions within the cytoplasmic inclusions. Note difference in morphology compared with (B). ×70,000. (441) (Nazerian)

herpesvirus 1 (serotype 3). Serotype 1 MDV is the prototype virus for this group of avian viruses, and except where otherwise indicated, MDV refers to serotype 1 virus. On the basis of their virulence, serotype 1 strains are further divided into pathotypes, which are often referred to as mild (m)MDV, virulent (v)MDV, very virulent (vv)MDV, and very virulent plus (vv+)MDV strains (699, 708).

Two additional groups of nononcogenic herpesviruses isolated from chickens (63, 143) and turkeys (338, 718), respectively, are considered part of the MDV group and are included in this chapter. The serotypic classification for MDV and HVT strains (675, 676), originally based on the recognition of common and distinct antigenic epitopes for each serotype, has recently been justified based on the complete sequence for serotype 2 HPRS-24 and serotype 3 FC126 virus strains (11, 304, 347).

Morphology

The morphology and morphogenesis of MDV have been reviewed by Kato and Hirai (335) and Schat (564). In general, viral particles are typical of those described for other herpesviruses. Scattered virions are commonly seen in the nucleus and more rarely in the cytoplasm or extracellular spaces. Hexagonal nucleocapsids 85–100 nm in diameter and enveloped particles 150–160 nm in diameter may be seen in thin sections of infected cell cultures. Enveloped virus particles appearing as irregular amorphous structures and measuring 273–400 nm can be observed in negatively stained preparations of lysed feather follicle epithelium (FFE) (110). Thin-section preparations of the FFE revealed large numbers of cytoplasmic enveloped herpesvirus particles in keratinizing cells. The morphology of MD virions in cell cultures and FFE is shown in Fig. 15.1A.

The morphology of serotype 2 and 3 strains resembles that of MDV serotype 1. In thin sections, however, nucleocapsids of HVT commonly show a unique crossed appearance (441). The morphology of serotype 2 MDV has not been studied in detail, but typical herpesvirus particles have been visualized (485, 571).

Chemical Composition

Viral DNA

Physical Properties

The complete sequence for the 3 serotypes (11, 304, 347, 375, 664) confirmed that the genomes are very similar consisting of linear, double-stranded DNA molecules of approximately 160–180 kb with a buoyant density in neutral CsCl of 1.706 g/mL for serotype 1 (368, 550). The guanine plus cytosine (G+C)

Table 15.2. Genomic structure of the 3 serotypes of Marek's disease viruses.

Genome organization[A]	Serotype and virus strain (Reference)			
	Serotype 1		Serotype 2	Serotype 3
	GA (375)	Md5 (664)	HPRS-24 (304)	FC126 (11,347)
%G+C[B]	43.9	44.1	53.6	47.6
	Number of nucleotides			
Total	174,040[C]	177,874	164,270[C]	159,160 (11) 160,673 (347)
α-type		963	ND[D]	251
TRL	12,585	13,065	11,825	5,658
UL	113,476	113,563	109,933	111,868
IRL	12,579	13,065	11,825	5,658
α-type		879	660	251
IRS	12,120	12,264	8,959	13,303
US	11,160	10,847	12,109	8,617
TRS	12,120	12,264	8,959	13,303
α-type		965	ND[D]	251

[A] The following abbreviations are used: UL = unique long sequence, TRL = terminal repeat flanking UL, IRL = internal repeat flanking UL, US = unique short sequence, IRS = internal repeat flanking US, TRS = terminal repeat flanking US.
[B] See references (304, 401).
[C] Does not include α-type sequences.
[D] ND = not determined.

ratio is different for the 3 serotypes and ranges from 43.9–53.6% in serotype 1 and 2, respectively, and 47.6% for HVT (Table 15.2) (304, 621). It is difficult to separate viral DNA from host cell DNA because its density is close to that of chicken DNA. Several methods have been described to obtain viral DNA (550). Pulse-field electrophoresis is probably the best way to obtain pure infectious viral DNA (269, 298, 683). However, the cloning of the complete MDV genome as bacterial artificial chromosomes (BAC) has greatly facilitated the production of MDV DNA (588, 745). Infectious BAC clones of at least 4 different MDV strains—three avirulent and the highly oncogenic RB-1B—have been constructed. This has allowed rapid genetic manipulation of MDV genomes to identify various determinants associated with MD biology (137, 467). Similarly, the rescue of infectious MDV by transfecting overlapping cosmid clones of the Md5 strain of MDV into chicken embryo fibroblasts (CEF) (537) has allowed rapid manipulation of MDV DNA by site-directed mutagenesis and the generation of recombinant viruses to gain insights into gene functions (400, 622).

Structural Organization

The genomic structure of the 3 serotypes is typical for alphaherpesviruses as previously suggested (135) with a unique long (U_L) and a unique short (U_S) sequence. These unique sequences are flanked by sets of inverted repeat sequences: the terminal repeat long (T_{RL}), internal repeat long (I_{RL}), internal repeat short (I_{RS}), and terminal repeat short (T_{RS}), respectively. Alpha (α)-like sequences typical for alphaherpesviruses are located at the terminal ends of the T_{RL} and I_{RL} and between the I_{RL} and I_{RS} regions (11, 304, 348, 664) (Table 15.2), which can be variable in length (539). These a-like sequences are believed to be important for the cleavage and packaging of viral DNA into virions.

The complete genome sequences of four serotype 1 MDV strains are available up to now. These include Md5 (177874 base pairs, GenBank Accession number AF243438), GA (174077 base pairs, GenBank Accession number AF147806), BAC clone of Md11 (178632 base pairs, GenBank Accession number AY510475), and BAC clone of CVI988 (178311 base pairs, GenBank Accession number DQ530348). The genome structure and the sequences of these strains are very similar and the differences in the lengths are mostly caused by the changes in the copy numbers of the direct repeats in the repeat regions of the genome. Comparison between the vMDV GA and vvMDV Md5 strains shows very few structural differences between serotype 1 strains (621). The U_L regions of GA and Md5 are very similar in length and organization, but the U_S sequence of GA is longer than in Md5. The differences in the U_S and the flanking repeats result in the presence of 1 copy of small ORF2 (SORF2) in GA and a second SORF2-like gene in Md5. In addition SORF1 in Md5 is completely located in the repeats, but has a frame shift resulting in a truncated SORF1. The importance of these differences is not clear. Additional minor differences between strains can be expected. CVI988 BAC contains 14 copies of the 132-bp repeat, the expansion of which was once thought to be associated with viral attenuation (619, 622). Kaplan and Schat (333) reported differences in 2 of the 3 promoter regions for ICP4 between the GA,



15.2. Genetic organization of the GA strain of Marek's disease virus. (375) (Proceedings of the National Academy of Sciences) (L. F. Lee)

RB-1B, and CVI988 strains. *In vitro* assays suggest that these differences may influence the level of transcription of ICP4. Comprehensive analysis of the sequence of the T_{RL}/I_{RL} regions in the genomes of 13 strains of varying virulence has identified several single nuceleotide polymorphisms (SNPs) which loosely partition between attenuated and nonattenuated strains (634). The physical map of GA is presented in Figure 15.2.

The complete genome sequences of serotype 2 strain HPRS 24 (GenBank Accession number NC_002577) (304) and the serotype 3 HVT strain Fc126 (GenBank Accession numbers AF291866, AF282130) (11, 347) have also been determined. Inspection of the sequences of the 3 serotypes confirms that the genomes are colinear as had been suggested based on cross-hybridization studies with cloned fragments of the 3 serotypes (285, 465). The three serotypes differ substantially in their restriction endonuclease digestion patterns (225, 264, 554, 618) but share significant homology at the DNA level (11, 304, 347, 375, 664).

DNA Structure in Infected Cells. The structure of viral DNA in infected cells is dependent on the virus-cell interaction. Linear viral DNA can be found in nuclei of cells undergoing virus replication (135). It is still unknown how viral DNA is maintained in latently infected, nontransformed cells (426, 550). The status of DNA in transformed cells has been difficult to determine in part because a variable percentage of the transformed cells may undergo viral replication at any point in time, in which case linear

DNA can be detected. In addition, most of the studies on the structure of viral DNA have been done using established cell lines, which may have undergone additional selection. Initial reports indicated an absence of integration (652) or a mixture of integrated and episomal DNA (334, 544). The association of viral DNA with chromosomes by density gradient centrifugation and *in situ* hybridization supported the idea that integration may occur. More recently, Delecluse *et al.* (186, 187) showed that viral DNA can be integrated into primary tumor cells and tumor cell lines. Integration into primary lymphoma cells occurred randomly at multiple sites. In cell lines, integration occurred at 2–12 sites, which were characteristic for the individual cell lines. Integration sites were preferentially located at the telomeres of large- and mid-size chromosomes or in minichromosomes. Note that the terminal ends of the MDV genome contain host cell telomere-like sequences (348), which are thought to assist in the preferential integration of the viral DNA near to the telomeres of the host cellular DNA in latently infected cells (186).

Structural Changes by Recombination and/or Mutation. Spontaneous recombination among the 3 MDV serotypes under field conditions is probably a very rare occurrence, despite the fact that chickens vaccinated with all 3 serotypes frequently are superinfected with virulent MDV. Concomitant infections in the same tissue (142) and cells (447) have been reported. Only 1 case of a recombination between 2 serotypes has been reported when a virus was isolated from the MSB1–41C cell line with serotype 1 and 2 sequences. Cloning of the junction fragment showed that this was indeed a recombinant virus (269).

Serotype 1 strains quickly develop altered biologic characteristics upon serial passage *in vitro,* such as loss of oncogenicity (156), reduced expression of the so-called A antigen [glycoprotein C (gC)] (156), and decreased replication *in vivo* (576) indicating that spontaneous mutations may have occurred. The gradual evolution of pathotypes toward greater virulence and the changes in biologic properties of MDV during *in vivo* backpassage (692) further support the mutability of MDVs.

These biological changes are accompanied by several molecular changes, although it is not clear which molecular change correlates with a specific biological change. An expansion was found within the BamHI D and H fragments that are commonly associated with cell culture passage and attenuation in serotype 1 strains (221, 265, 623). This expansion was caused by a tandem amplification of direct 132 bp repeat (278, 406, 555). Other changes have also been described including the deletion of 400 bp in the BamHI A fragment of CVI988 clone C (988C) and 988C/R6 (278) and a deletion of 200 bp in the BamH1 L fragment of the vvMDV strain Md11 (683). Additional changes have been reported for CVI988 in the *meq* gene (378) and the ICP4 promoter/enhancer region of CVI988 (333). It is not clear whether these differences are a consequence of cell culture passage or reflect strain differences.

Serial passage of serotype 2 and serotype 3 strains can also lead to biological and structural changes. The biological changes are often associated with loss of *in vivo* replication and protective immunity (703, 719, 735). Other changes have also been reported such as the release of large quantities of cell-free HVT (735) or the development of resistance to inhibition by phosphonoacetate (371). Structural DNA changes have been described using restriction endonuclease patterns (267, 618) and total size estimates based on pulsed field electrophoresis experiments (683).

In vitro co-cultivation of MDV or HVT with avian leukosis virus (ALV) or reticuloendotheliosis virus (REV) resulted in spontaneous insertion of long terminal repeats (LTR) of the retroviral provirus in the MDV genome (295, 319), but full-length, infectious provirus occasionally can integrate (297). Integration of endogenous ALV sequences into MDV has also been reported (560). The integration occurs often at the border regions of the repeats and unique sequences of MDV and in the gD gene (80). Davidson and Borenshtain (173, 174, 175) reported the *in vivo* integration of retrovirus sequences into MDV in commercial poultry. The relevance of the integration of retrovirus for the pathogenesis of MD is not clear, although LTR insertions can cause increased transcription of MDV genes (318).

Viral Genes and Proteins

Over the last 20 years, a number of individual genes of MDV-1 have been identified and sequenced, and the proteins have been characterized (558, 720). Comprehensive reviews based on the complete sequences for the 3 serotypes recently have been published including lists of ORFs and their putative products (304, 401, 467, 621). Table 15.3 summarizes the location of the ORFs and indicates the number of ORFs with homologues to HSV, the number of ORFs with homologues shared among the 3 serotypes, and the number of unique genes for each serotype. Many of the genes in the U_L and the U_S regions have homologues with HSV and equine herpesvirus 1 and 4, and the genome organization is similar to these 2 alphaherpesviruses (401). For this chapter, the MDV genes are grouped into 2 general categories: genes with homologues in alphaherpesviruses and genes unique for MDV. Only the genes that are important for the pathogenesis and immune responses will be reviewed briefly. The reader is referred to the contemporary literature for additional information.

Genes with Homologues in Alphaherpesviruses

This broad category of genes can be divided into immediate early (IE), early, and late genes, which are with few exceptions important for virus replication.

IE and Early Genes with Homology to HSV. The IE genes are important transcriptional regulators. Four IE genes have been identified: intracellular protein (ICP)4, ICP0, ICP22, and ICP27. Anderson *et al*. (17) identified ICP4 as a 4245 bp ORF, but sequence data indicated the presence of an open reading frame (ORF) of 6969 bp. This is in agreement with the finding that 2 functional promoter/enhancer regions are located upstream of the larger ORF and that the putative promoter/enhancer region for the short ORF was nonfunctional in *in vitro* assays (333). Proof that ICP4 protein is a transactivator was provided by transfection of the MD cell line (MDCC) MSB-1 with the short form of ICP4, showing increased transcription of the pp38 and pp24 genes (520) and the endogenous ICP4 gene (206, 520). The ICP4 pro-

Table 15.3. Number of tentative genes in the 3 serotypes of MDV in relation to other alphaherpesviruses[A].

		Location of expected functional ORFs[B]						
Serotype	**Gene classification**	**TRL (R-LORF)**	**UL (L-ORF)**	**IRL (R-LORF)**	**IRS (RS)**	**US (S-ORF)**	**TRS(RS)**	**Total[C]**
1	HSV Homolog[D]	0	57	0	1	7	1	*65*,**66**
	MDV-specific[E]	1	4	1	0	1	0	*6*,**7**
	Serotype-specific	13[H]	8	13	2	3[H]	2	*26*,**41**
	Total	14	69	14	3	11	3	*97*,**114**
2	HSV Homolog	0	59	0	1	7	1	*67*,**68**
	MDV-specific[F]	1	4	1	0	1	0	*6*,**7**
	Serotype-specific	9	4	9	1	4	1	*17*,**27**
	Total	10	66	10	2	12	2	*90*,**102**
3	HSV Homolog	0	59	0	1	8[I]	1	*68*,**69**
	MDV-specific[G]	0	6	0	0	1	0	*7*,**7**
	Serotype-specific	4	2	4	6	1	6	*13*,**23**
	Total	4	67	4	7	10[I]	7	*88*,**99**

[A] For reference see (11, 304, 375, 401).
[B] Based on the location of the start codon.
[C] The italic numbers indicate the number of single genes for each serotype; the bold figures give the total number of genes including the duplications in the repeat regions.
[D] Based on the sequence of the GA strain, nomenclature adapted from (340).
[E] Serotype-specific genes with homologues present in serotype 2 or 3.
[F] Serotype-specific genes with homologues present in serotype 1 or 3.
[G] Serotype-specific genes with homologues present in serotype 1 or 2.
[H] The sequence for Md5 has minor differences compared with GA.
[I] Includes 2 copies of US8.

tein can also transactivate the LTR of Rous sarcoma virus at low levels (41). Transcription of ICP4 may require the presence of VP16, a late protein coded for by UL48 and present in the tegument of MDV particles (72). However, Kaplan and Schat (333) were unable to confirm this observation, and the requirement for VP16 in a cell-associated virus may not be absolute.

The role of ICP22 (=US1) protein (81) in MDV replication is poorly understood. Deletion mutants lacking ICP22 replicate *in vitro* but to a lower degree than wild-type virus. ICP22 is not essential for *in vivo* infection, oncogenesis, and reisolation of virus (472, 473). The MDV ICP27 phosphoprotein (540) localizes in the nucleus, can transactivate pp38 and pp14 independently of ICP4, and represses the early thymidine kinase gene (541). Although the precise functions of the MDV ICP27 have not been demonstrated, recent reports on the role of HSV ICP27 in the mRNA export pathway (352) and NFκβ activation (250) suggest important roles for this protein in MD biology. ICP0 (R-LORF1) has been identified as an ORF in the TRL and IRL, and a recent study using proteomic approaches has demonstrated that the ICP0 gene product is expressed in MDV-infected CEF (393). However, it is currently unknown if the protein has similar functions as ICP0 in HSV.

The early genes with homology to HSV-1 have been identified. The functions of these genes are expected to be similar to those in other alphaherpesviruses (401) and will not be further discussed in this chapter. The MDV genes MDV008 and MDV073 encoding the related phosphoproteins pp24 and pp38, respectively, (664) with early expression kinetics are discussed separately.

Late Genes. The late gene products include the nucleocapsid proteins, the tegument proteins, including VP16, and the glycoproteins (reviewed in 401). The glycoproteins (gB, gC, gD, gH, gI, gK, gL, and gM) will be discussed briefly because these are presumed to be important for infection of cells, transfer of virus from cell to cell, and immune responses. Churchill *et al.* (156) identified 2 glycoproteins by AGP tests: the soluble A antigen and the cell-bound B antigen, which are now known as gC and gB, respectively.

gB, encoded by UL27, consists of a complex of 3 glycoproteins with molecular weights of 100, 60, and 49 kD (gp100, gp60, and gp49) (138, 292, 299, 449, 620). gB is important for cell attachment and/or penetration based on the production of gB-specific virus-neutralizing (VN) antibodies (292, 299, 442, 463, 552). Deletion of gB from MDV prevented the cell to cell spread demonstrating the essential nature of this protein for MDV replication (588), as is the case with all other herpesviruses (500).

The UL44 gene encodes gC, a 57–65 kD glycoprotein identified in some early references as gA, which is extensively synthesized in productively infected cells and is expressed on the cell surface and cytoplasm. In addition, gC is actively secreted by infected cells (165, 290, 296, 300) and is one of the major antigens to which the chicken immune system mounts a substantial serological response. The function of gC has not been elucidated; the production of gC in cell culture decreases with attenuation (156, 291) probably due to reduced transcription of UL44 (684). Recent data suggest that the reduction of MDV replication due to overexpression of gC in cultured cells is possibly caused by the

secreted forms of gC (659). A gC deletion mutant had an attenuated phenotype with a decrease in infectivity, horizontal transmission, and oncogenicity. However, a revertant virus needs to be generated to confirm that the attenuation was caused by deletion of gC (422). Recent *in vivo* studies using a gC-negative mutant of RB-1B showed that although the viral load in the peripheral blood was not affected, it was compromised in establishing latency and inducing tumors suggesting a major role for gC in MD pathogenesis (468).

The importance of gD, coded by US6, is poorly understood. It is expressed poorly (464) or not at all (651) *in vitro* probably as a consequence of no or limited transcription. Limited expression of gD compared to pp38 and gB has been described in FFE (450), suggesting that specific transcription factors in the FFE may be needed for the production of gD. A gD deletion mutant constructed in the oncogenic RB-1B strain was fully oncogenic and capable of horizontal transmission indicating that gD is nonessential (18). Recently SNP analysis in the gD gene was used in a retrospective molecular epidemiological study to determine the variation among MDV strains (364).

The functions of the other glycoproteins have not been studied in detail. The gI and gE proteins interact with each other based on immunoprecipitation assays (651). It is not clear whether the gE/gI complex functions as Fc receptors as described for other alphaherpesviruses. Mutants constructed in a BAC clone carrying deletions in the gM, gI, or gE gene indicate that the encoded glycoproteins are essential for virus replication, because the deletion mutants are unable to transfer infectivity from infected to uninfected cells (589, 660).

Genes Unique for MDV

Several genes have been identified that are unique for MDV strains (Table 15.3). Some of these genes are present only in serotype 1, and others may have homologues in MDV serotype 2 and/or HVT.

Latency Associated Transcripts (LATs). The LATs are a group of transcripts antisense to ICP4 and recently have been reviewed in detail (426). These include a large 10 kb transcript as well as several spliced transcripts referred to as MSR (MDV small RNA) or SAR (small antisense RNA) (132, 133, 387, 388, 417). The importance of LATs for latency or transformation is unclear. LATs are expressed in both lytically infected and transformed cells. One of the small LATs, identified as SAR, was expressed consistently in the $CD4^+$, $AV37^+$ fractions of primary lymphomas (557). LATs have also been described in the MDV-positive QT35 cell line (736). A deletion mutant of RB-1B with an insertion of LacZ, which disrupts the 5′ end of the MSR has been generated. Chickens inoculated with the mutant virus were able to induce robust lytic infection but failed to induce tumors. The importance of this finding is not clear until a rescued virus has been generated from this mutant (426).

Meq (Marek's EcoQ). The molecular biology of Meq (R-LORF7) has been reviewed (360, 426, 435). The Meq protein of 339 amino acids contains a basic leucine zipper (bZIP) domain at the N terminal closely resembling the *jun*/*fos* oncogene family. The proline-rich repeat region at the C terminal resembles the WT-1 tumor suppressor gene (320). The Meq protein is expressed consistently in the nucleus of lymphoma cells and tumor cell lines (396, 532, 557) and can be expressed during the S-phase (394). Meq has a domain for dimerization with itself or the cellular oncoprotein Jun; the complex can bind to two distinct sequences, MERE I and MERE II. MERE I is located in the promoter/enhancer region of *meq*, and a MERE II site has been located in the putative MDV origin of replication (531, 532). Several lines of evidence suggest that Meq is important for transformation. The expression of *meq* antisense RNA in MSB-1 cells reduced colony formation in soft agar (732). Overexpression of Meq in transfected rat cells resulted in morphological transformation, inhibition of apoptosis (396), and interactions with the cell cycle regulator CDK2 (397). Interestingly, CVI988 has a 178 bp insert resulting in a frame-shift encoding for the proline-rich domain (378), although the frame-shift in the protein sequence could not be confirmed in a subsequent study (502). Forms of large Meq (L-Meq) containing the insertion have a suppressive effect on the Meq expression on the basis of reporter assays (136). Variations in the sequence of the proline-rich domains also showed association with virulence (594).

Meq shows differential binding to different promoters depending on its dimerization status. As heterodimers with leucine zipper proteins, e.g., c-Jun, Meq can transactivate promoters containing AP-1 sites (385) resulting in the upregulation of a number of genes including interleukin (IL)-2 and CD30, a member of the tumor necrosis factor receptor II (TNFR-II) family (93). It is suggested that Meq transforms chicken cells through the activation of genes such as JTAP-1, JAC, and HB-EGF, all of which are associated with the v-Jun transforming pathway (384). These data together with the upregulation of anti-apoptotic factors such as Bcl-2 and c-*Ski*, the cellular homologue of the retrovirus-transduced oncogene v-*Ski*, strongly indicate a converging pathway for transformation by oncogenic retroviruses and herpesviruses. Further evidence for a direct role for Meq has come from a recent study in which a Meq-deletion mutant of the vvMDV strain Md5 failed to induce tumors. However, this may also be caused by a significant reduction in the replication of the virus. Specific interaction between Meq and the transcriptional co-repressor protein CtBP was shown to be critical for MD oncogenicity, since specific mutations abolishing the interaction resulted in the total loss of oncogenicity of the virus (78).

Two spliced products from the Meq region have also been identified, one of which has been named vIL-8, and the second one lacks the transactivator domain. Examining the dynamic cellular properties and distribution of Meq and Meq/vIL8 proteins Anobile *et al*. (22) indicated that these two forms may have fundamentally different functions in MDV-infected cells. In addition to Meq, two transcripts of 852 and 1168 bp antisense to *meq* have been described. The 1168 bp ORF codes for a 23 kd nuclear protein that has been detected in lytically infected and transformed cells (495).

v-IL8. Recently, a homologue of the avian chemokine IL-8 has been identified in MDV (395, 475). The vIL-8 gene (R-LORF2)

is located in the long repeat region and originally was identified by Peng and Shirazi (494) as a spliced *meq* variant. The gene consists of 3 exons and is expressed late during cytolytic infection. IL-8 attracts T cells, especially after IL-8 receptors are upregulated by interferon-γ (IFN-γ) leading Schat and Xing (584) to hypothesize that vIL-8 may be important for the switch of infection from B to T lymphocytes (see "Pathogenesis").

Viral Lipase. The 3 serotypes of MDV code for a viral lipase gene (v-LIP) (11, 304, 347, 375, 664). v-LIP, a soluble, glycosylated protein, is encoded by the R-LORF-2 gene consisting of 2 exons. The first exon codes for the signal peptide, and the second exon codes for the lipase activity. v-LIP is probably an IE or early protein (328). The glycosylated protein is required for the efficient lytic replication in birds (329).

pp38/pp24. The MDV phosphorylated protein complex, often referred to as pp38/pp24, is coded by 2 genes located at opposite ends of the U_L region (749). The pp24 gene (R-LORF14) is located partly in the TR_L and the U_L region, and the pp38 gene (R-LORF14a) is located in the IR_L and the U_L region. Homologues for pp24 and pp38 have been identified in serotype 2 strains (304, 466). The TR_L and IR_L of HVT contain a gene with homology to pp38, but the functional relationship to serotype 1 pp38 is unknown (11, 626). The presence of these homologues was expected because HVT and SB-1 induce cell-mediated immune response to pp38 (460, 521).

The function of the pp24/pp38 complex has not been elucidated. Originally, it had been linked to oncogenicity because pp38 is expressed in the cytoplasm of a variable proportion of MDV-transformed, latently infected lymphocytes (169, 288, 437, 438). Expression of pp24/pp38 can be enhanced by treatment with IUdR (289) or transfection with the ICP4 gene (520). Interestingly, infection of QT35 cells, which are latently infected with serotype 1 MDV, with HVT activates pp38 expression (736). These data suggest that pp38 may play a role during reactivation and subsequent virus replication rather than with oncogenicity. The fact that pp38 is also expressed in productively infected cells including the FFE (169, 288, 437, 438) supports this hypothesis. Ross (558) suggested that pp38 may be responsible for the induction of apoptosis. A recent study examining the pp38 expression in QT35 cells identified two new splice variants that enhanced the metabolic activity suggesting additional roles for this phospoprotein in MDV latency and transformation (389). Recently it has been shown that pp38 is essential for cytolytic infection of B cells and maintenance of transformed state (536). However, pp38 deletion did not affect the ability of the virus to spread horizontally (229). Minor differences in amino acid sequences among strains have been noted for pp38. Originally, it was believed that pp38 was not expressed in CVI988 (725). However, it subsequently was shown that the gene is present in CVI988 but that amino acid 107 in an epitope defined by monoclonal antibody (MAb) H19 was changed from glutamine to arginine (170, 171). The biological relevance of this difference is not clear. The demonstration that vvMDV5 strain expressing the pp38 protein from CVI988 remains oncogenic indicates that the attenuation of CVI988 is not associated with pp38 (367).

The promoter/enhancer regions of pp38 and pp24 are part of a bidirectional promoter complex regulating the transcription of pp38/pp24, and the 1.8 kb gene family. This region also contains the origin of replication (423). Several transcription factors are located in this region including binding sites for Meq. Interestingly, serotype 2 has also a binding site for Meq (MERE II) in this region (614), although a homologue for the *meq* gene has not been identified in HPRS-24 (304). Differential transcription for pp38/pp24 and the 1.8 kb gene family has been reported (614).

The 1.8 kb Gene Family. Several immediate early transcripts originate from the 1.8 kb gene family containing 3 exons (73, 354, reviewed in 426). These transcripts are truncated in attenuated strains due to an expansion of a tandem 132 bp direct repeat (132 bp DR) (74, 555). Usually, nonattenuated serotype 1 strains including low passage CVI988 have few copies, and attenuated strains have multiple copies of the 132 bp DR (278, 331, 556), a distinction that forms the basis of differentiation by polymerase chain reaction (PCR) assays (49, 616, 750). However, the 132 bp region *per se* appears not to be directly associated with oncogenicity since the deletion of the region did not appear to affect the pattern or the frequency of tumors (622). Furthermore, it was shown that viruses lacking the 132 bp repeats can still be attenuated by repeated cell culture passages (619).

So far, a 7KDa (493) and a 14 KDa (277) protein have been associated with these transcripts. Both proteins can be detected in lytically infected and transformed cells, but the 7 KDa protein was not found after infection with attenuated virus. The function of these proteins has not been elucidated, although expression of 1.69 kb and 1.5 kb cDNAs derived from the 1.8 kb gene family in transfected CEF prolonged proliferation and reduced serum dependence, suggesting that one of the functions may involve the control of the cell cycle (492). In addition, expression of oligonucleotides antisense to the 132 bp DR transcripts inhibited proliferation of lymphoblastoid cell lines (339), further suggesting that the 1.8 kb gene family may be important for transformation.

Telomerase RNA (vTR). The existence of a unique gene encoding the RNA telomerase subunit (vTR) was identified in the IR_L/TR_L region of the MDV genome (217). MDV vTR showed nearly 88% sequence identity to the chicken telomerase RNA (ChTR) indicating its transduction from the host genome. vTR can constitute telomerase activity by interacting with chicken telomerase reverse transcriptase (ChTERT) more efficiently than ChTR (218). The direct association between MDV oncogenicity and vTR was recently demonstrated using RB-1B virus lacking either one or both copies of vTR (662). vTR-negative mutants were significantly impaired in their ability to induce lymphomas with smaller less-disseminated tumors.

MDV-encoded microRNAs. MicroRNAs (miRNAs) are a distinct class of small regulatory molecules of approximately 22 nt affecting the gene expression in various cell types. These have been identified in a large range of organisms including several herpesviruses (436). Recently, several novel MDV encoded miRNAs flanking the Meq gene and the LAT region of the genome were

identified in MDV-infected CEF (95). The precise functions of these novel miRNAs in MD biology are not yet known. However, as these molecules are expressed at very high levels in MD lymphomas and MDV-transformed cell lines, they may play major roles in oncogenesis.

Other Unique Genes. Proteins have not been identified for several unique ORFs that are transcribed in tumor cells. Most of these have not been further studied with a few exceptions. RLORF5a [ORF-L1 (456)] is expressed in tumor cell lines, QT35 latently infected with MDV, and in a REV cell line latently infected with MDV (456, 496, 736). The function of RLORF5a remains unknown. Expression is not essential for reactivation from latency and virus replication (580) or tumor formation (313).

Jarosinski *et al.* (312) found that RLORF4 was expressed in MD tumor cell lines and that this ORF was deleted in a series of attenuated MDV strains. Deletion of both copies of RLORF4, but not one copy, in RB-1B resulted in an attenuated phenotype *in vitro* and resulted in a major reduction in tumor development, but early viral replication was not affected by the deletion (313).

Viral Vectors

Several nonessential sites in the 3 serotypes of MDV can be used for the insertion and expression of foreign and specific MDV genes (reviewed in 268). The anticipated advantages of MDV-vectored vaccines are that these vaccines will protect simultaneously against MD and other pathogens, and reactivation from latency will reinforce immune responses against MD and the other pathogens. So far, most of the MDV-vectored vaccines have shown protection in specific-pathogen-free (SPF) chickens [e.g., against Newcastle disease (424) and very virulent infectious bursal disease (IBD) (663)]. The disadvantage is that the MDV-vectored vaccines need to be given *in ovo* or at hatch. The presence of maternal antibodies against the expressed foreign protein may impede the development of active immunity against the inserted gene and perhaps also MDV, especially if the inserted gene is under the control of a strong promoter. For example, when the F protein of Newcastle disease virus (NDV) was expressed in a CVI988 MDV-vectored vaccine under control of the SV40 late promoter, protection against NDV challenge was suboptimal in maternally antibody-positive chickens. However, expression under control of the MDV gB promoter induced protection (633).

Virus Replication

Replication of the 3 serotypes is typical of other cell-associated herpesviruses and has been reviewed extensively (58, 335, 467, 550, 564). For initial infection of cultures or chickens by cell-free virus, enveloped virions bind to cellular receptors probably by gB perhaps in combination with other glycoproteins. Heparan sulfate, a member of the glycosaminoglycans, has been identified as one of the cellular receptor molecules (377). In cell cultures, viral penetration occurs within 1 hour after attachment, which is enhanced for serotype 1 by chelators such as ethylenediaminetetraacetic acid (EDTA) (8). Subsequent spread of infection to other cells occurs by direct contact with infected cells, and virus transfer probably is accomplished through formation of intracellular bridges (327). This is presumed to be the principal mode of virus spread after initial infection *in vitro* and *in vivo*. Recent data demonstrate the role of the U_S3-encoded kinase in the morphogenesis as well as cell-to-cell spread of virions through the effect of stress on fiber breakdown and polymerization of actin (590). The glycoproteins gE, gI, and gM play a role in the transfer of virus from infected to uninfected cells (589, 660). Replication rates vary with serotype, passage level of the virus strain, cell type, and temperature of incubation.

The spread of virus *in vivo* from cell to cell will require intimate contact between infected and uninfected cells, which are most often lymphocytes, although epithelial cells also can be involved in this process. The precise interaction between these cells remains one of the important unsolved issues.

Virus-cell Interactions

Three general types of virus-cell interactions are recognized: productive, latent, and transforming.

Productive Infection. During productive infection, replication of viral DNA occurs; proteins are synthesized; and in some cases, virus particles are produced. The number of genome copies per cell can increase 100-fold and exceed 1200 in the case of HVT (334). Two types of productive infection exist. Fully productive infection in the FFE of chickens results in development of large numbers of enveloped, fully infectious virions (110). In productive-restrictive infection, most of the virions are nonenveloped and noninfectious. A variable number of the virions in cultured cells may be enveloped, however, and these can be recovered as cell-free, infectious virus by disruption of cells. The use of an appropriate stabilizer such as SPA (116) will improve the yield of cell-free virus. A variant strain of HVT that releases large quantities of cell-free virus into the medium of infected cell cultures has been described. This strain is replication-defective in chickens (735). In all susceptible cells, productive infection leads to intranuclear inclusion body formation and lysis of the cell. A gene for the viral host shut-off protein has been identified, UL41 (401), that is probably responsible for the initiation of the lytic process. Lytic infection *in vivo* can cause frank necrobiotic lesion formation. Because of this, productive infection has been termed cytolytic, and the terms are used synonymously (105).

In productively infected fibroblasts, most of the MDV genome is transcribed (407, 570, 625). Differences in transcripts between productive infection with virulent and attenuated serotype 1 strains have been described (73, 555) and are mostly associated with transcripts in the repeat regions flanking U_L. Recent study employing a mass spectrometry-based proteomic approach has further confirmed the expression of the majority of MDV-encoded proteins during cytolytic infection in CEF (393).

Productive infection in cell cultures is influenced by several factors. Inhibitors of viral DNA polymerase and thymidine kinase will inhibit virus replication in productively infected cell cultures but do not influence the growth of lymphoblastoid cell lines (550, 564). The presence of nitric oxide (NO) in cell cultures reduces virus replication in a dose-dependent manner (188, 734). *In vitro* infection can change transcriptional regulation of

cellular genes in infected and in neighboring cells. Microarrays have been used to determine which genes may be up- or downregulated by infection with MDV (425). For example, MHC class I and II genes and two IFN-response elements were upregulated. The importance of these results needs to be further examined. Hunt *et al.* (281) reported that productive-restrictive MDV replication downregulated expression of MHC class I expression in OU2 cells and in lymphoblastoid cell lines. Upregulation of MHC genes actually occurs in noninfected cells (340) probably through IFN (383). This is certainly compatible with the upregulation of IFN-response genes. IFN, probably IFN-α, may be produced at least by some MDV and HVT strains (276, 326).

Latent Infection. Latent herpesvirus infections have been defined as the presence of viral DNA in the absence of viral transcripts and proteins, although LATs have been described for many herpesviruses. This definition is appropriate for the nontransforming serotype 2 and 3 strains. For serotype 1 strains, the distinction between latency and transformation is often problematic. In both cases, the viral genome is present, but no information is available on differences in transcriptional regulation between latently infected and transformed cells, because it is impossible to separate latently infected, nontransformed cells from noninfected cells. As a consequence, studies on latency have often been done in MD transformed cell lines.

MDV latency mostly is associated with $CD4^+$ T cells, although $CD8^+$ T cells and B cells can also be latently infected (125, 376). Fewer than 5 copies of the viral genome are present in latently infected cells (550). *In vitro* latent infections have been described in REV-transformed (522), OU2 (6) and QT35 cell lines (736). The MDV genome can be reactivated from latently infected cells and tumor cells by inoculation of susceptible chickens, cocultivation with permissive cells, and *in vitro* cultivation of latently infected lymphocytes. The latter approach can be used to estimate the number of latently infected cells by enumeration of antigen-positive cells at 0 hours and 48 hours in culture (127). The presence of latent MD infections can also be detected by PCR assays (see "Diagnosis"). However, the PCR assay needs to be combined with RT-PCR, demonstrating the absence of early and late transcripts to ensure that the amplified DNA is obtained from latently and not from the few productive-restrictive infected cells that commonly occur in populations of latently infected lymphocytes. Upregulation of ICP4 transcripts and downregulation of LATs have been associated with reactivation of early and late genes in latently infected cells (6, 736).

At least 2 cytokines produced by concanavalin A-stimulated spleen cell cultures can help maintain latency in cultured lymphocytes (96). Subsequently, 2 recombinant chicken (rCh) cytokines, rChIFN-α (671) and rChIFN-γ (332), have been shown to suppress production of IE, early, and late viral antigens in latently infected lymphocytes. Curiously, IFN-α was more effective in the suppression of viral genes in later stages of latency than during early stages (671). *In vivo* selective reactivation of latently infected cells can be induced by treatment with cyclosporin or betamethazone (97), but not by infection with immunosuppressive viruses, such as IBD virus (IBDV) or REV. Chicken infectious anemia virus (CIAV) may influence latency in MD based on its influence on cytokine production and cytotoxic T lymphoctes (CTL) (567).

Transforming Infection. Transforming infections occur only in cells infected with serotype 1 MDV. Selection of transformed cells from the background of immunologically committed and noncommitted cells (490) would facilitate comparative studies on transformed cells in tumors and tumor cell lines. The search for specific surface markers associated with tumors has yielded 2 potential antigens. An MD tumor-associated surface antigen (MATSA) was detected on cells from MD lymphomas and lymphoblastoid cell lines but not on the surface of productively infected cells (511, 731). MATSA was also detected on lymphocytes from chickens vaccinated with HVT or serotype 2 strains of MDV (349, 512, 572), and subsequent studies revealed MATSA to be present on activated T cells from uninfected chickens (412). Recently, a second antigen, CD30, detected by MAb AV37, has been associated with MD-transformed $CD4^+$ T cells and MDV-infected cells during the cytolytic phase. However, this antigen can also be detected on B cells and REV-transformed cells (92, 557). Recent studies confirm that $CD30^{hi}$ expression is characteristic of MD lymphomas suggesting that CD30 is a component of a critical intracellular signaling pathway perturbed in neoplastic transformation (93). Both MATSA and CD30 can be used to enrich for transformed cells in tumor cell suspensions. Ross *et al.* (557) purified cells from lymphomas using CD4 and AV37 MAbs and found that *meq* and SAR transcripts were abundant in these populations. In contrast, pp38 and VP16 transcripts were not detected. Most other studies have used lymphoblastoid cell lines to identify transcripts, which have been detailed in a previous section. Antigens normally are not detected in lymphomas or lymphoblastoid cell lines by FA tests with convalescent serum, except for occasional cells that probably have converted to a productive infection (12, 119) and, by definition, are no longer transformed. Some transformed lymphocytes can be induced to produce viral antigens by treatment with IUdR (128, 195, 366) or by culture at suboptimal temperatures (23, 128).

Replication of Other Serotypes

In most reports, replication of MDV serotype 2 and HVT are similar to serotype 1. Because HVT and serotype 2 MDV are nononcogenic (571, 718) no cell lines have been developed equivalent to those derived from MD lymphomas, and transforming infections have not been recognized.

Latent infections have been demonstrated in chickens infected with serotype 2 and 3 viruses (613). The phenotype(s) of cells latently infected with serotype 2 and 3 strains have not been elucidated. The observation of latent infection of SB-1 in ALV-transformed B cell lines suggests that B cells are likely target cells for latent infection with serotype 2 strains (223). Occasionally, HVT can be present in MDV-transformed T cell lines, probably representing a latent infection (119, 266).

Holland *et al.* (274) reported the presence of HVT transcripts encoded in the TR_L and IR_L in the thymus and spleen, but not the bursa of Fabricius, in the absence of gB expression. Interestingly,

these transcripts were also detected in peripheral nerves and feather-associated tissues.

Virus Stock Production and Stability

Productively infected cell cultures are a common source of cell-associated virus stocks for all 3 viral serotypes and for cell-free HVT stocks. Techniques for the production and cryopreservation of cell-free and cell-associated virus stocks have been described (reviewed in 153). Cell-associated stocks of MDV or HVT are routinely stored at –196°C. The infectivity of such stocks, however, is directly related to viability of the cells contained in these preparations and depends also on optimal freezing and thawing techniques. Under ideal conditions, the half-life of diluted, cell-associated virus stocks or vaccines should be at least 2–6 hours (656).

Cell-free serotypes 1 and 2 virus stocks are best obtained from FFE (low-passage virus) or infected cell cultures (high-passage virus). Small quantities of low-passage virus can be obtained from infected cell cultures by lysing cells in SPA (116). The production of cell-free HVT is best achieved by lysing heavily infected cell cultures. Cell-free MDV and HVT can be stored at –70°C or lyophilized (116). Potency of both cell-associated and cell-free vaccines can be affected adversely by storage temperature, reconstitution technique, choice of diluent, and holding time and temperature after reconstitution (245, 478).

Susceptibility to Chemical and Physical Agents

The stability of cell-associated MDV serotype 1 and 2 strains is completely dependent on the viability of the cells. Any treatment affecting cell viability will impact directly the infectivity of virus stocks.

Cell-free MDV obtained from the skin of infected chickens was inactivated when treated for 10 minutes at pH 3 or 11 and stored for 2 weeks at 4°C, 4 days at 25°C, 18 hours at 37°C, 30 minutes at 56°C, or 10 minutes at 60°C (109). Dander, litter, and feathers from infected chickens are infectious and presumably contain cell-free virus from the FFE bound to cellular debris. The infectivity of such materials was retained for 4–8 months at room temperature (271, 705) and for at least 10 years at 4°C (102). Virus infectivity was inactivated by a variety of common chemical disinfectants within a 10-minute treatment period (115, 270). Survival of virus in litter may be affected adversely by increased humidity (705).

Strain Classification

Serotypes

Von Bülow and Biggs (675, 676) classified the MDV herpesvirus group into 3 distinct virus groups that correlated with biologic properties. Type-specific monoclonal antibodies (286, 370) usually are used to determine virus serotype.

Although distinguishable by serologic tests, the 3 serotypes also share many common antigens. Thus, sera against a serotype usually will react with antigens of other serotypes, although somewhat less vigorously than with homologous antigens (675).

A number of biological characteristics are associated with viral serotypes (63, 564). Low-passage serotype 1 viruses grow best in duck embryo fibroblast (DEF) or chicken kidney cell (CKC) cultures, grow slowly, and produce small plaques. Serotype 2 viruses grow best in chicken embryo fibroblasts (CEF), grow slowly, and produce medium plaques with some large syncytia. HVT grows best in CEF, grows rapidly, and produces large plaques. More infectious virus can be extracted from HVT-infected cells than from cells infected with serotype 1 or 2 viruses.

Pathotypes

Virulence or oncogenicity is associated with only serotype 1 MDVs. Within this group, however, a wide variation in pathogenic potential is recognized and undoubtedly represents a continuum from nearly avirulent to maximally virulent. Pathotypic classification schemes have evolved over the last 30 years with the continued increase in virulence. Current classification schemes recognize 4 groups of viruses. These groups are designated as mMDV, vMDV, vvMDV, and vv+MDV (699, 708). Pathotyping of virus isolates involves comparative pathogenicity tests in vaccinated and unvaccinated maternal antibody positive chickens with prototype viruses as controls (693, 708). No *in vitro* methods have yet been developed. Prototype viruses are the CVI988 (546) and CU2 (627) strains of mMDV; the JM (593), GA (197), and HPRS-16 (527) strains of vMDV, the Md5 (721) and RB-1B (574) strains of vvMDVs; and the RK-1 (113), also identified as 625 by Witter (699), and the 648A (699) strains of vv+MDV. The evolution in the virulence of MDV strains is recognized, but the molecular basis for this evolution has not been elucidated. For many years, MD was the classic disease with paralysis as the main lesion induced by viruses of the mMDV pathotype. A more virulent form of MD was first noted in the late 1940s (50), associated with viruses of the vMDV pathotype, which became the dominant pathotype during the 1960s. The vvMDV pathotype virus strains were first noted in the late l970s (196), mainly in HVT-vaccinated flocks with excessive MD losses, which led to introduction of bivalent vaccines in the early 1980s. During the early 1990s, the vv+ strains appeared and together with the vv strains are the dominant types.

Certain biologic characteristics are associated with pathotypes of serotype 1 MDVs but are most pronounced between low-passage and high-passage (attenuated) strains. Serial passage *in vitro* (30–70 passages usually are required) results in attenuation of virulent isolates (156, 546, 576, 688). Attenuated strains grow more readily *in vitro* but produce lower viremia titers *in vivo* (712), which may be associated with a marked decrease in their ability to infect and/or replicate in lymphocytes (576). The production of gC (A antigen) is reduced or absent (156). Attenuated strains do not spread well among chickens by contact (184, 692). Some strains are incompletely attenuated and induce minor lesions in highly susceptible chickens (505, 674). Overattenuated strains do not replicate in or protect chickens (353, 719). The *in vivo* growth potential of attenuated serotype 1 isolates can be increased by backpassage in chickens (185, 692), although in one case virulence was also increased (185). The incidence of tumors induced by low-virulence strains of serotype 1 MDV is increased

by infection at 7 days of embryonation or by immunosuppression (112, 121). Viruses of serotype 2 (SB-1 strain) and 3 remained nononcogenic following similar treatments (121, 571).

Laboratory Host Systems

MDV usually is propagated and assayed in tissue cultures, newly hatched chicks, and embryonated eggs. Lymphoblastoid cell lines from MD lymphomas are also an important laboratory host system.

Cell Cultures

The propagation of MDV serotypes *in vitro* has been reviewed in (58, 564). Cultured DEF or CKC prepared from 1- to 2-week-old chicks are suitable for isolation and propagation of low-passage MDV isolates (154, 631). Isolation of low-passage MDV in CEF or embryonal CKC cultures is far less efficient than in CKC or DEF (569). Propagation in CEF leads to accelerated attenuation compared to CKC or DEF (576). In embryonal CKC, replication of serotype 1 MDV (but not HVT) is abortive, leading to loss of infectivity within 2 to 3 passages. Attenuated MDV and serotype 2 and 3 viruses can be isolated readily and propagated in CEF (63, 571). Infected cultures usually develop discrete focal lesions, called foci or plaques, which consist of clusters of rounded, refractile degenerating cells when mature (Fig. 15.3). Plaques are usually less than 1 mm in diameter and of variable cell density, although plaque size varies with viral strain, time, and other factors. Polykaryocytosis is seen in cultured fibroblasts and is a major component of the viral plaques or foci frequently used as a marker in virus assays. Affected cells may contain 2 to several hundred nuclei, and type A intranuclear inclusion bodies are commonly seen. Despite release of rounded cells into the medium as plaques mature, large areas of cell lysis are not seen.

Serotype 1 plaques develop in 5–14 days on primary isolation and in 3–7 days after adaptation to culture and usually are enumerated by microscopic examination, but different staining techniques have been developed, allowing enumeration at a later time. Differences in development and morphology of serotype 1 plaques in chick and duck cells and in plaques induced by the 3 viral serotypes (564) have been described. Other cell culture systems such as chick embryo skin (518), tracheal explants (535), and embryo fibroblasts from several avian species including Japanese quail (528) also have been used.

A few avian cell lines have been used for the propagation of MDV strains. The OU2 cell line can be used to propagate the three serotypes. Plaques develop after the monolayers become stationary, but virus remains latent when the cultures are subconfluent (5, 7). The DF-1 cell line can also be used for the propagation of all three serotypes of MDV. Recently developed quail cell lines free of MDV can be used to propagate serotype 1 MDV and HVT, but SB-1 did not replicate efficiently (390).

Serotype 1, but not serotype 2, MDVs can also be grown in chicken splenic lymphocytes *in vitro* (126). Passages are made by the addition of fresh spleen cells to the suspension cell cultures every 2 days, and infection is monitored by immunofluorescence. HVT may be similarly grown in turkey spleen cell cultures, but viral antigen is rarely seen, if at all.

Mammalian cell lines and primary cultures are, in general, considered to be refractory to infection (reviewed in 530, 564). However, a recent report suggests that Vero (African green monkey kidney) cells can replicate MDV and HVT, but the possible survival of inoculum-derived CEF or chicken-Vero cell hybrids has not been excluded (308).

Chickens

Newly hatched chicks inoculated with virulent, serotype 1 MDV develop gross lesions or lesions that can be detected histologically in ganglia, nerves, and certain viscera after 2–4 weeks. Response is greatly dependent on genetic susceptibility of the chicken and virulence of the MDV isolate. Presence of virus or antibody, which can be detected by *in vitro* tests, or the presence of virus-associated antigen detected by FA tests on tissues, are also specific host responses of inoculated chickens to MD infection. All these responses are markedly enhanced in chicks lacking maternal antibodies against MDV (100). The induction of virus-specific lesions in the wing web (118) or the feather pulp (427) constitutes alternate approaches that provide direct access to the site of lesion development.

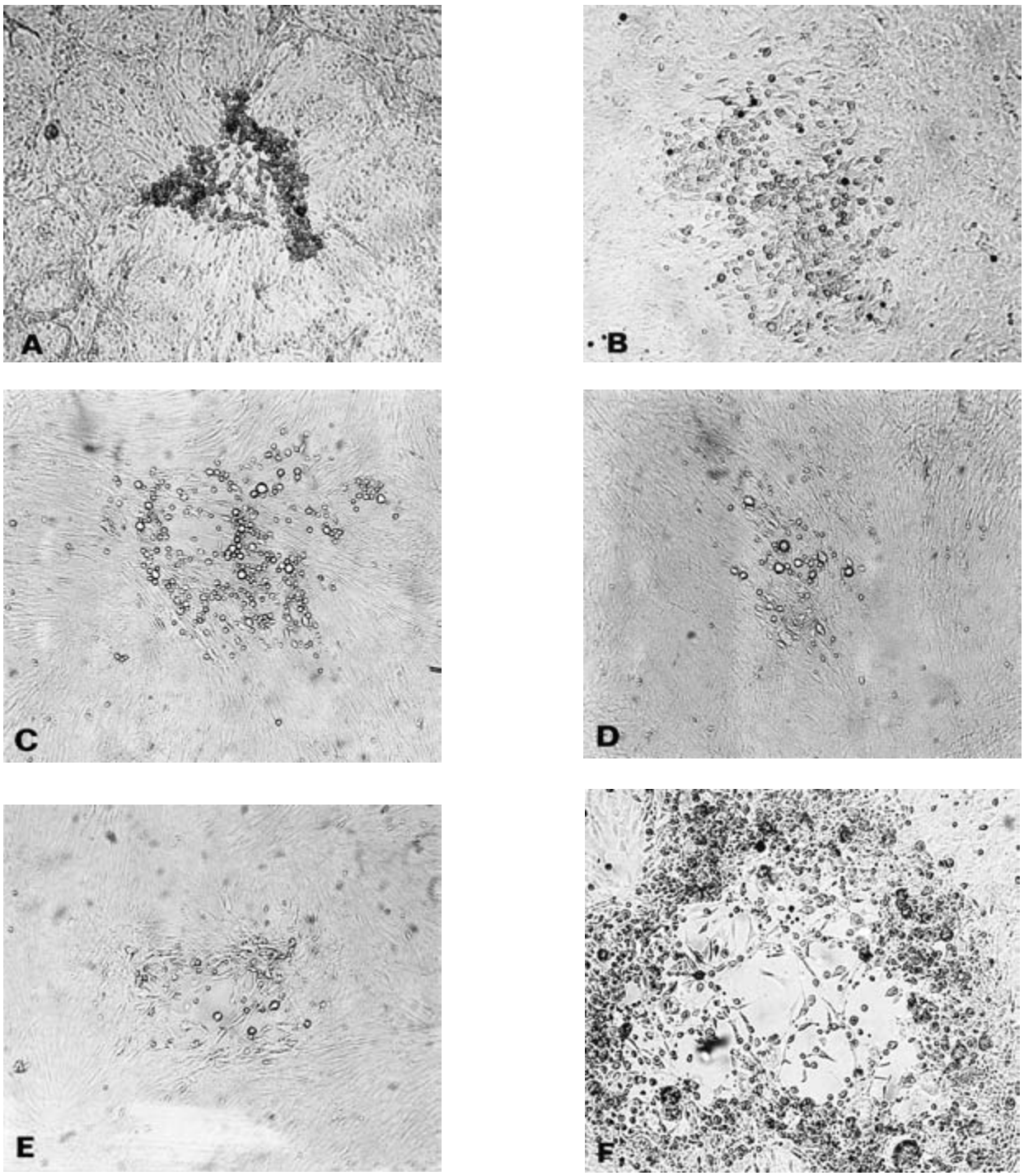
Embryos

Virus pocks develop on the chorioallantoic membrane (CAM) of chicken embryos following yolk sac inoculation with cellular MDV preparations (62, 673). Embryos have also been used for MD vaccine evaluation, because *in ovo* vaccination is becoming increasingly common in the field (See "Pathogenesis of *in ovo* Infection"). Embryos may also be used to isolate MDV viruses that cannot be isolated directly in cell culture for unknown reasons. Yamaguchi *et al.* (736) reported the isolation of MDV from the QT35 cell line by using kidney cell cultures prepared from 4- to 7-day-old chicks that had been inoculated at ED 8 with QT35 cells.

Lymphoblastoid Cell Lines

Lymphoblastoid cell lines developed from MD lymphomas (see 106) grow continuously in cell culture without attachment to the culture vessel. Success rates for establishing cell lines from MD lymphomas have improved because of better methodology (128, 486) but *in vitro* immortalization of lymphocytes has not been achieved with 2 exceptions (120, 287). Many cell lines are now available including several from MD lymphomas in turkeys (439). The majority of the chicken cell lines established from lymphomas are $CD4^+/CD8^-$ T cells expressing MHC class II and T cell receptor (TCR) 2 or 3 (474, 577). Lymphoblastoid cell lines can also be established from lymphocytes harvested from early [4–6 days postinfection (PI)] lesions induced in the wing web or pectoral muscle by injection of a mixture of MDV and allogeneic kidney cells. The cell lines from early lesions may be $CD4^+/CD8^-$, $CD4^-/CD8^+$, or $CD4^-/CD8^-$ (577, 579). Cells of the MDCC-RP1 line are illustrated in Fig. 15.4.

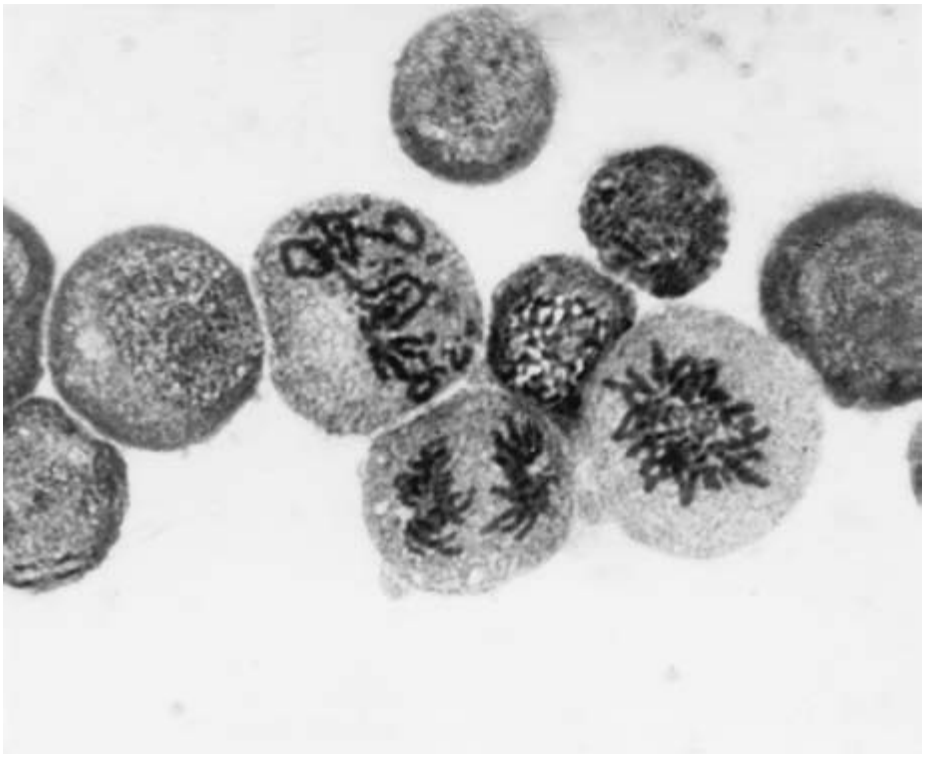
Some transformed cells contain about 5–15 copies of viral genome, although the mean number may be considerably higher in different cell lines, perhaps in relation to the proportion of productively infected cells in the population (426, 550). Viral DNA



15.3. Focal lesions in cultured cells infected with various Marek's disease virus (MDV) serotypes. A. Low-passage serotype 1 MDV in chicken kidney cells cultured from an infected chicken, 9 days. B. Low-passage serotype 1 MDV in duck embryo fibroblasts (DEF), 5 days. C. High-passage, attenuated serotype 1 MDV in chick embryo fibroblasts (CEF), 5 days. D. Low-passage serotype 2 MDV in CEF, 8 days. E. Low-passage HVT (serotype 3) in CEF, 4 days. F. Low-passage turkey herpesviruses (HVT) in DEF, 12 days. All photos unstained, about ×40. (Witter)

can be highly methylated in cell lines in contrast to viral DNA in productively infected cells (330). However, methylation is not essential for maintaining the transformed state (474).

Most cell lines can be termed "producer" lines, because a small proportion (1–2%) of the cells enter into productive infection (128, 511). Virus can be recovered readily from most cell lines, although several nonproducer cell lines have been developed in which evidence of genome expression is limited or lacking (444, 486, 652). Prolonged culture can result in reduced expression of the MDV genome (411). Most, but not all, MD cell



15.4. Smear from the MDCC-RP1 cell line. Note the characteristic lymphoblastoid morphology and the mitotic figures. Giemsa, ×1500. (Nazerian)

lines established from lymphomas were found to display a chromosomal aberration in which an amplification of DNA resulted in an extra G-band and interband in the short arm of chromosome 1 (70, 421). The aberration was found only infrequently in MD lines established from local MD lesions (420), and it remains to be determined whether any relationship exists between this change and neoplastic transformation.

MD cell lines have been used to analyze the potential interaction with tumor suppressor genes and cellular oncogenes. Meq protein induces the transcription of the proto-oncogene *bcl-2* in Rat-2 cells (396), and the gene product can delay apoptosis. However, Ohashi *et al.* (455) were unable to detect the transcription of *bcl-2* in 2 cell lines and in T cells 3 weeks after infection *in vivo*. The transcript for *bcl-xL*, another gene involved in the prevention of apoptosis, was expressed, suggesting the *bcl-xL* rather than the *bcl-2* gene product may be important for transformation. Several mutations of p53, a tumor suppressor gene, have also been reported, but these mutations are not located in the traditional hot-spots associated with loss of function of p53 (647, 649). However, several kinds of truncated p53 transcripts with truncations ranging from 101–765 bp, thought to be generated by alternate splicing, were identified in MD-derived tumor cell lines (648). Further studies examining the expression of p53 protein identified two forms—a 40kDa large form and a 30kDa small form—of the p53 protein in MD-transformed cell lines (650). The levels of the short form of the protein showed increase during apoptosis suggesting that this form could play a role in the initiation of apoptosis.

Recently, a fibroblast cell line has been established that was apparently transformed by MDV. This cell line produces pp38, Meq, and the late gene product gB, but virus particles were not demonstrated (88). It is unknown whether complete viral DNA is present in these cells.

Pathobiology and Epidemiology

Multiple Syndromes

It is now apparent that MD consists of several distinct pathologic syndromes (107). Also, differences are apparent among the types of MDV-induced syndromes typically seen in layer/breeder flocks, broiler flocks, and those induced in the laboratory. Of the various syndromes, lymphoproliferative syndromes are most frequently associated with MD and have the most practical importance (see Table 15.4A). Of these, MD lymphoma is probably the most common. However, fowl paralysis, persistent neurological disease, skin leukosis, and ocular lesions are additional clinical manifestations that have lymphoproliferative components. Some of the lymphoproliferative syndromes may also have degenerative components. Several additional clinical syndromes characterized solely by degenerative and inflammatory lesions, often with accompanying immunosuppression, are induced by MDV infection in the laboratory (see Table 15.4B). Nonneoplastic brain pathology, mainly vasogenic edema, is responsible for transient paralysis (231). Vascular lesions are manifested as atherosclerosis. Under laboratory conditions, young chicks inoculated with tumor cells may develop localized or diffuse transplantable tumors (641, 655). Inoculation of MDV-infected, allogeneic CKC in the wing web may induce what Calnek *et al.* (118) termed local lesions. Some of the syndromes induced under laboratory conditions are rare or nonexistent in the field, probably because

Table 15.4A Clinical and pathologic syndromes associated with Marek's disease virus (part A).

	Lymphoproliferative syndromes[AB] (Marek's disease)			
Situation in which syndrome observed	**Lymphomas and nerve lesions**	**Fowl paralysis (nerve lesions)**	**Skin leukosis (integument)**	**Blindness and ocular lesions**
Experimental chickens (laboratory)				
Clinical signs	Depression, death, stunting, paralysis	Paralysis	Swollen feather follicles	Blindness, ocular lesions
Mortality	0–100%[C]	0–30%[CD]	None	Rare or none[C]
Age	Onset 2–8 wk PI	Growing birds	Young birds[E]	4–8 wk PI
Organ	Visceral organs + peripheral nerves	Mostly peripheral nerves	Skin	Eye (iris, cornea)
Layer/Breeder flocks (field)				
Clinical signs	Depression, death, paralysis	Paralysis, death	Swollen feather follicles	Blindness, gray eye
Prevalence	Common	Occasional[D]	Rare or none[E]	Rare
Mortality	0–60%	0–20%	None	None
Age	4–90 wk	8–20 wk	4–8 wk PI	>10 wk
Broiler flocks (field)				
Clinical signs	Depression, death, paralysis	Paralysis, death	Swollen feather follicles, red leg	Blindness, gray eye
Prevalence	Common	Rare or none[D]	Common[E]	Rare or none
Mortality	Minor		None	None
Age	At processing		At processing	

[A]Neoplastic lesions may include inflammatory components.
[B]Severity of syndrome usually less in vaccinated flocks.
[C]Depends on experimental conditions (virus strain, dose, chicken genotype, maternal antibody status, prior vaccination, etc.)
[D]Rarely induced by contemporary MDV strains, except in conjunction with visceral neoplastic lesions.
[E]Not usually recognized except at broiler processing or after feather removal.

Table 15.4B Clinical and pathologic syndromes associated with Marek's disease virus (part B).

	Lymphodegenerative syndromes	**CNS syndromes**	**Vascular syndromes**	**Other syndromes**
Situation in which syndrome observed	**Early mortality syndrome, cytolytic infection, immunodepression**	**Transient paralysis and persistent neurological diseases**	**Atherosclerosis**	**Local lesions; transplants**
Experimental chickens (laboratory)				
Clinical signs	Depression, stunting, death, increased disease susceptibility	Transient paralysis, tics, torticollis, death	None	Swelling at inoculation site
Mortality	0–100% [AB]	0–100%[AB]	None	Yes (transpl.)
Age	9–20 days PI	9–28 days PI	Adult birds	Young birds
Organ	Bursa, thymus, spleen	Brain	Blood vessels	Web—local and many—transpl.
Layer/Breeder flocks (field)				
Clinical signs	Increased disease susceptibility	Transient paralysis, tics, torticollis		N/A: Only experimental
Prevalence	Rare[A]	Rare[A]	Rare or none	
Mortality		Rare		
Age		5–12 wk		
Broiler flocks (field)				
Clinical signs	Increased disease susceptibility	Transient paralysis, tics, torticollis		N/A: Only experimental
Prevalence	Rare[A]	Occasional[A]	None	
Mortality		Rare		
Age		5–7 wk		

[A]Not normally observed in chickens vaccinated for MD.
[B]Depends on experimental conditions (virus strain, dose, chicken genotype, maternal antibody status, prior vaccination, etc.).

most commercial chickens are hatched with passive MD antibodies and receive MD vaccines at or before hatch.

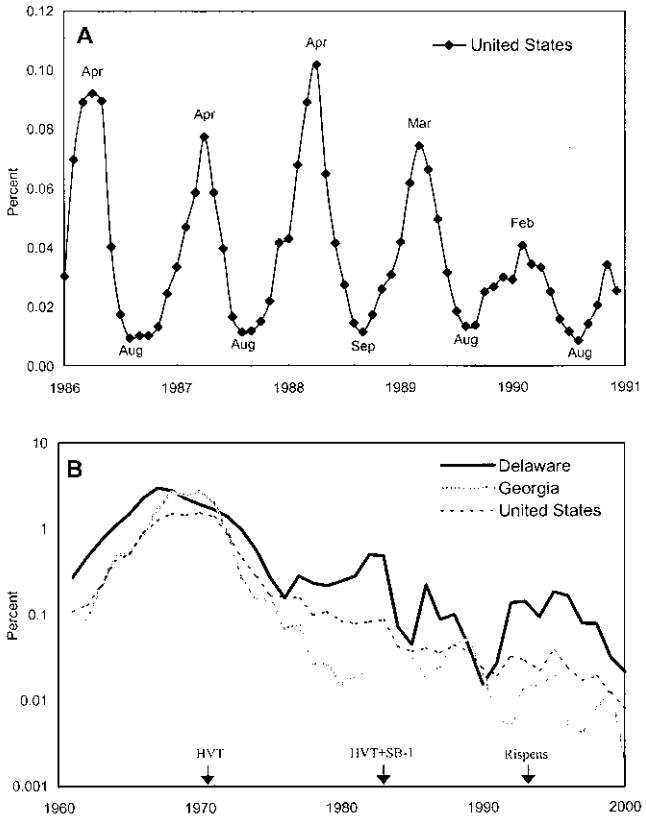
Subclinical disease syndromes may also occur but are more difficult to define. Purchase *et al.* (529) found that vaccinated flocks produced more eggs than nonvaccinated flocks, indicating that MDV may depress productivity in otherwise normal-appearing, nonvaccinated chickens.

Incidence and Distribution

MD exists in poultry-producing countries throughout the world. Probably every flock of chickens raised in areas where poultry is prevalent becomes infected, and many experience some loss. Reporting systems vary, however, and it is difficult to determine the true incidence. The incidence of infection is surely much higher than the incidence of disease. Even in susceptible chickens, infection does not always induce clinical disease and, in genetically resistant or vaccinated chickens, infection may rarely cause overt disease.

In the United States, the incidence of MD losses can be illustrated through the use of broiler condemnation data, collected since 1961 at the processing plant. A biphasic pattern is noted consistently with maximum frequencies around April and minimum frequencies around August (Fig. 15.5A), perhaps reflecting the effects of temperature stress and limited ventilation during winter months (526). Regional differences are striking. Analysis of the devastating 1960s outbreak showed that the initial losses occurred in the Delmarva and northeastern states, but the disease spread quickly to other regions (698). Historically and to the present time, condemnations in Delaware have been consistently higher and condemnations in Georgia have been consistently lower than mean values for the United States (Fig. 15.5B). Long- and short-term trends due to the introduction of vaccines can also be evaluated through condemnation data. Since 1971, when HVT vaccine was introduced, through 2000, yearly condemnations have decreased by 79-fold, 169-fold, and 958-fold in Delaware, the United States, and Georgia, respectively (Fig. 15.5B). Upturns in the early 1980s and the early 1990s were both successfully resolved by the introduction of more effective vaccines (see "Vaccination"). However, during the early part of 2005 outbreaks occurred in the Delmarva region with condemnations of almost 10% in broilers vaccinated with bivalent and trivalent vaccines (561). Layer and breeder flocks have experienced similar trends.

Sporadic outbreaks of MD occur on individual farms or regions. Several reports lend credence to the implication of exceptionally virulent MDV isolates in vaccine failures (196, 510, 574, 721). It is usually difficult to associate increased virulence of MDV isolates with regional fluctuations in MD frequency, but layer outbreaks in northwestern Ohio in 1995 (399) yielded several isolates of unusual virulence (113, 699).



15.5. Marek's disease condemnations in young broilers. A. Monthly averages illustrating biphasic annual pattern (United States). B. Annual averages 1960–2000 for Delaware, Georgia, and the United States. Approximate times of introduction for major vaccine strains are indicated with arrows. (Data from National Agricultural Statistics Service.) (Witter)

Natural and Experimental Hosts

Chickens are by far the most important natural host for MD, but quail, turkeys, and pheasants are also susceptible to virus infection and disease. Virtually all chickens including game fowl (344), native breeds (235, 559) and jungle fowl (145, 681) are susceptible to MDV infection and tumor development. Most other avian species including ducks, sparrows, partridge, pigeons, and peafowl (46, 234, 342, 513) are probably refractory, although MDV-inoculated ducks developed antibodies (46). Mammals, including several species of primates, are also refractory to experimental inoculation (155, 272, 546, 609, 611). Mice inoculated with tumor cells from chickens from field cases of MD developed a high rate of neoplasms (16), but the etiological role of MDV in these tumors is unsubstantiated.

Quail

In Japanese quail, natural outbreaks of MD in commercial flocks are relatively common (350). Affected birds develop lymphomas in various visceral organs, but peripheral nerves are rarely affected (350, 497, 516). Mortality can reach 10–20%, but deaths occur relatively late. MDV antigens can be demonstrated in the FFE, and MDV occasionally can be isolated from blood, although titers can be low (279, 293) or detected by PCR assays (497). Transmission of MDV from chickens to quail and from quail to chickens has been reported (294). MD can be induced in quail by experimental inoculation or contact exposure to MDV strains of both chicken and quail origin (293, 345). The experi-

mental disease appears similar to that of natural outbreaks. Many commercial quail flocks are vaccinated with HVT, and Kobayashi *et al.* (350) considered vaccination to be protective. However, Kaul and Pradhan (337) reported that quail were poorly protected by HVT against challenge at 10 days of age with an MDV strain of quail origin. Bobwhite quail are also susceptible, but perhaps less so than Japanese quail (513).

Turkeys

In turkeys, MD-like tumors have been occasionally reported (21, 679), but causal associations with MDV usually have been lacking, and natural outbreaks have been rare, at least until recently. However, turkeys are susceptible to experimental infection with MDV with mortality around 22–70% at 8–19 weeks (179, 479, 730). Enlarged peripheral nerves were rare or absent in these studies. Characteristics of the viral infection were similar to those in chickens but generally are more subdued. Virus could be reisolated from peripheral blood lymphocytes and antigen detected in lymphoid tissues and lungs of inoculated turkeys but less frequently and at lower concentrations than in chickens (201). Immunosuppression was also noted in turkeys that developed tumors (201). Interestingly, vaccination with HVT provided no protection against MDV challenge (202). Both B-cell (440) and T cell (509) lines have been developed from turkey lymphomas induced by MDV inoculation, suggesting that both cell types are susceptible to transformation.

Severe clinical outbreaks of MD in commercial turkey flocks were reported in France (164), Israel (179), Germany (670), and Scotland (498). Mortality from tumors reached 40–80% between 8–17 weeks of age. In some of these cases, the affected turkey flocks were raised in proximity to broilers. The lesions were similar to those produced experimentally, although paralysis was noted in some birds, and peripheral nerves were occasionally infiltrated with lymphocytes (164). Serotype 1 MDV was detected by virus isolation or PCR (176). Transmission between turkeys and between chickens and turkeys was established (164). Vaccination of turkeys with CVI988 appears to offer protection (164). The disease has not yet shown the ability to become endemic within commercial turkey populations.

Other Species

Pheasants and related species like the Black Francolin may also be susceptible as indicated by occasional reports of typical lymphomas and nerve lesions (251, 322, 503). Challenge of the common pheasant (*Phasianus colchicus*) with virulent MDV induced paralysis, visceral lymphomas, and precipitating antibodies within 75–85 days PI (380). Unlike quail and turkeys, pheasants appear to be more susceptible to neurological lesions of MD. However, the disease has not been well characterized in this species. MD was reported in white-fronted geese (*Anser albifrons*) in Japan (661). Subsequent analysis showed high prevalence of MDV genome in the feather tips of this species (433).

Transmission, Carriers, Vectors

MDV is transmitted readily by direct or indirect contact between chickens, apparently by the airborne route (citations in 57). Epithelial cells in the keratinizing layer of the feather follicle replicate fully infectious virus (110), and these cells serve as a source of contamination to the environment and to other chickens. Virus associated with feathers and dander is infectious (48, 130), and contaminated poultry house dust remains infectious for at least several months at 20–25°C and for years at 4°C (citations in 105). Under commercial field conditions, young chickens are most commonly exposed to MDV by contact with residual dust and dander in the growing house or by the introduction of these materials by aerosols (from adjacent chicken houses), fomites, or personnel. After the virus is introduced into a chicken flock, regardless of vaccination status, infection spreads quickly from bird to bird. Virus excretion begins about 2 weeks after inoculation or exposure (341), with maximal shedding occurring between the third and fifth week (686). Once infected, chickens appear to shed virus indefinitely (729). Quantitative (q)PCR assays have recently been established to measure virus load in FFE and dust for MDV serotype 1 including the vaccine strain CVI988 (2, 34, 39, 301), serotype 2 (543) and serotype 3 (301). Preliminary data confirm the earlier observations that maximal shedding occurs early after the presence of virus in the FFE.

Darkling beetles (*Alphitobius diaperinus*) were shown to passively carry the virus, but free-living litter mites, mosquitoes, and coccidial oocysts could not be associated with transmission (47, 75, 198). Vertical transmission of MDV does not occur (546, 630, 632). Transmission from dam to progeny as the result of external egg contamination is also unlikely because of poor virus survival at temperature and humidity levels used during incubation (115).

Experimental transmission is accomplished by inoculation of day-old, genetically susceptible chicks with blood, tumor suspensions, or cell-free virus by virtually any parenteral route. Exposure by direct or indirect contact with infected chickens is also effective. The virus can be transmitted by intratracheal instillation or inhalation exposure using cell-free virus preparations. Cellular inocula contain few, if any, infectious virions and are unlikely to cause infection except by parenteral inoculation.

Incubation Period

The incubation period for experimentally induced MD is well established (see reviews 32, 107). Mononuclear infiltrations may be found in nerves and other organs after about 2 weeks (488). Clinical signs and gross lesions, however, generally do not appear until between the third and fourth weeks (484).

The incubation periods can be short for several nonlymphomatous syndromes associated with MDV infection. Cytolytic infections occur at 3–6 days PI and are followed by degenerative lesions (atrophy) of the thymus and bursa of Fabricius within 6–8 days PI (107). The early mortality syndrome is characterized by deaths at 8–14 days PI (721). The clinical expression of both acute and classical forms of transient paralysis usually occurs from 8–18 days PI (343, 710). Field cases of transient paralysis are seen mainly between 6–12 weeks of age, probably reflecting MDV exposure 8–10 days prior to the onset of symptoms. Development of atherosclerosis requires 3–7 months (209). Induction of tumors within 10–14 days after inoculation of cellular material is suggestive of a transplantation response (641).

Local lesions in the wing web are visible 3–4 days PI with allogeneic MDV-infected CKC (118).

Under field conditions, MD outbreaks sometimes occur in unvaccinated layer chickens as young as 3–4 weeks. Most of the serious cases begin after 8–9 weeks but also sometimes commence well after the onset of egg production (358). MD outbreaks in commercial, vaccinated chickens have been termed as "early" and "late" breaks, referring to the apparent failure of vaccines to provide protection (703). The late breaks are especially troublesome and have been known to occur late in the laying cycle (446), or even after chickens have been induced to molt and the second laying cycle has commenced. It is uncertain whether late onset of disease is caused by early (old) or late (recent) infection. Witter and Gimeno (709) infected birds between 18 and 102 weeks of age and found that nonvaccinated birds developed MD within 68 days PI when challenged with highly virulent strains. In contrast, vaccinated birds of similar ages did not develop MD after challenge. The authors suggested that late breaks were not likely the result of recent infections alone and that additional factors are needed to cause the late breaks.

Clinical Signs

Signs associated with MD vary according to the specific syndrome (Tables 15.4A and 15.4B). Chickens with MD lymphoma or fowl paralysis syndromes may exhibit signs, but few are specific to MD (55). In general, signs related to peripheral nerve dysfunction are those associated with asymmetric progressive paresis and, later, complete spastic paralysis of one or more of the extremities. Involvement of the vagus nerve can result in paralysis and dilation of the crop and/or gasping. Because locomotory disturbances are easily recognized, incoordination or stilted gait may be the first observed sign. A particularly characteristic clinical presentation is a bird with one leg stretched forward and the other back as a result of unilateral paresis or paralysis of the leg (Fig. 15.6). However, chickens with MD lymphomas may exhibit few signs and, instead, become depressed and comatose prior to death. Other chickens may appear clinically normal and still have extensive neoplastic involvement when euthanized. Nonspecific signs such as weight loss, paleness, anorexia, and diarrhea may be observed, especially in birds in which the course is prolonged. Under commercial conditions, death often results from starvation and dehydration because of the inability to reach food and water or, in many cases, from trampling by penmates. Some birds develop nervous tics or torticollis 18–26 days after virus exposure, often after recovery from classical transient paralysis. This syndrome has been termed persistent neurological disease (231) and can be induced by partially attenuated MDVs that no longer induce transient paralysis (230). However, the central nervous system (CNS) signs are difficult to distinguish from those associated with MD nerve lesions.

Birds with ocular involvement may show evidence of blindness (214), which can be unilateral or bilateral, although recognition of clinical blindness requires careful observation. Affected eyes gradually lose their ability to accommodate to light intensity.

Early mortality syndrome results in high mortality 8–16 days PI of young chickens with virulent MDV strains (667, 721). Chickens become depressed and comatose prior to death, which occurs within 48 hours of the onset of signs. Some affected chickens may also exhibit flaccid neck paralysis prior to death (710). Chickens undergoing acute cytolytic infection at 3–6 days PI may be depressed but rarely die during this period, although some may die later from early mortality syndrome. Immunosuppressed chickens may succumb to ancillary infections, but some chickens die 20–40 days PI with few signs.



15.6. Fowl paralysis. Spastic paralysis of limbs associated with peripheral nerve involvement in Marek's disease. (Witter)

A transient paralysis syndrome has been described in field flocks (741) and is associated with MDV infection (343). However, it has been observed infrequently in the field since vaccination for MD has become widespread. Two forms exist, classical and acute. In the classical form, affected chickens display varying degrees of ataxia and flaccid paralysis of the neck or limbs beginning 8–12 days after exposure to the virus by inoculation or contact (Fig. 15.7). Signs typically last 1–2 days followed by a rapid and complete recovery, although recovered chickens may succumb a few weeks later with MD lymphomas. The acute (fatal) form results in death within 24–72 hours following the onset of paralytic signs (710).

Morbidity and Mortality

The incidence of MD is quite variable in commercial flocks. Although a few birds that develop signs apparently recover from the clinical disease (65, 89), the recovery is rarely permanent; chickens that develop clinical disease usually die. Prior to the use of vaccines, losses in affected flocks were estimated to range from a few birds to 25 or 30% and occasionally as high as 60%. In broilers, MD condemnations averaged 1.0% in 1970 and could reach 10% or higher in individual flocks. Presently, nearly all commercial egg-type chickens are vaccinated against MD, and this has reduced losses to less than 5% in most countries (526). Broiler flocks, which are vaccinated in some but not all countries, may experience mortality of 0.1–0.5% and condemnations of 0.2% or more (526). The average condemnation rate for MD in the United States has decreased dramatically (Fig. 15.5B), falling to less than 0.001% in most regions in 2002 (432). However, spikes in condemnations can occur as was noted in Delmarva in 2005 (561).

Some flocks experience significant disease outbreaks despite



15.7. Transient paralysis. Flaccid paralysis of neck of young chicken 9 days after inoculation with Marek's disease virus. (Courtesy of *Avian Diseases*) (Witter)

vaccination. After the disease appears, mortality builds gradually and generally persists for 4–10 weeks. Outbreaks occur in isolated flocks or occasionally in several flocks in a region or in succeeding flocks on a farm. An interesting, albeit fortunate, tendency has developed for regional outbreaks to abate spontaneously. The reasons for the occurrence and cessation of regional outbreaks are poorly understood.

Response rates or mortality approaching 100% for lymphomas, early mortality syndrome, acute cytolytic infection, or transient paralysis can be achieved following inoculation or exposure of unvaccinated, susceptible chickens to MDV. Because the response frequency is influenced by many factors (see below), laboratory experiments can be designed to produce a wide range of specific clinical and pathologic responses.

Factors That Influence Mortality and Lesions

Virus Strain

The virulence of MDV strains varies widely and appears to have increased over time (699). Compared to the milder forms of the disease, which caused mainly peripheral nerve lesions, the more virulent MDV pathotypes more frequently induce higher mortality and more visceral lymphomas, and have the tendency to more frequently break through genetic host resistance or immunity induced by vaccination (66, 721). In some outbreaks, a high incidence of blindness and ocular lesions appeared to be related to only certain virus strains (214, 638). However, in other trials, many contemporary MDV strains appeared to induce ocular lesions (699). The extent of disease induced by a given strain depends in part on the genetic constitution of the host (575).

Virus Dose and Route

Dosage may influence disease frequency under natural conditions, although the MD response in genetically susceptible birds given virulent virus was found to be maximal even when a limiting dilution of virus was inoculated (628). Route of exposure probably functions in the same manner because less efficient routes may effectively decrease the dose to the bird.

Host Gender

Biggs (56) cited several studies in which it was observed that females died earlier and experienced higher losses than males. The difference was apparently not due to sex hormones, varied with the genetic strain, was most pronounced with susceptible strains of chickens, and was most apparent with viruses of higher virulence. In practice, the influence of gender appears variable and is probably less important than other factors.

Maternal Antibody

Maternal antibody reduces and delays MD mortality (151) and virtually all other manifestations of the disease, probably by limiting the spread of virus in tissues during the first few days post exposure (99, 488). Transient paralysis can be reproduced consistently only in chickens lacking maternal antibodies (343). The early mortality syndrome is reduced markedly in chickens with maternal antibodies (721). Breeder stocks are vaccinated uniformly and exposed to virulent MDV, and, therefore, virtually all chickens are hatched with maternal antibodies (normally against multiple viral serotypes). SPF flocks are a source of antibody-free chicks for laboratory studies. Thus, unlike some other virus infections, passive antibodies do not provide a sterilizing immunity, and antibody-positive chickens can be infected successfully and vaccinated, albeit with reduced responses.

Host Genetics and Age at Exposure

Genetic factors and age at initial exposure are important determinants of MD susceptibility (see reviews 25, 86). Genetic resistance correlated with the development of virus-neutralizing antibody (99, 607) and retention of cell-mediated immune functions (372, 582). This resulted from a sparing of immune competence in the case of resistant birds, rather than from an inherent difference between strains (259, 260, 627).

Newly hatched chicks and older chickens are both susceptible to infection and cytolytic infection (107), but in older chickens, cytolytic infections are resolved more rapidly (97) and virus load is somewhat lower (181). The frequency of lymphomas is variable and often markedly reduced in older chickens compared to newly hatched chicks, especially in genetically resistant lines (19, 101, 668, 724). However, nonvaccinated, SPF, older chickens may develop high rates of lymphomas and transient paralysis following challenge with vv and vv+ strains (548, 709). Age-related resistance can be abrogated by neonatal thymectomy (610) suggesting that other immunosuppressive stress factors may increase the susceptibility of older chickens to disease, at least under some conditions. Lesion regression has been identified as the basis for age-related resistance (608).

Prior Infection

Before the use of vaccines, naturally occurring infections with serotype 2 MDV often provided some protection against later exposure to oncogenic strains (307). Mild strains of serotype 1 MDV also can induce protective immune responses (628). However, the influence of natural infections with avirulent serotype 1 MDVs on disease response in vaccinated chickens probably is

minimal and, at present, such infections are not likely to have much clinical importance.

Environmental Factors and Stress

Various environmental factors and intercurrent infections appear to affect the incidence of MD, probably through interference with host immune responses. Gross (237) observed increased incidence among chickens selected for high concentrations of plasma corticosterone or subjected to a high degree of social stress. The administration of corticosteroids to latently infected chickens precipitated the appearance of clinical MD (507) and the feeding of corticosteroid inhibitors tended to increase resistance to the disease (160). Restricted feed intake delayed and reduced incidence of MD (247) whereas high-protein diets (524) or the selection of chickens for fast growth rate were associated with increased susceptibility to the disease (246).

Effects of intercurrent infections with other avian pathogens on MD have been extensively studied. Because MDV infection may depress host immune responses in its own right, concurrent infections are often exacerbated. Examples include coccidiosis (61) and cryptosporidiosis (1, 434). However, when the concurrent infection is itself immunosuppressive, the resulting immunosuppression usually will exacerbate both disease processes. Examples include IBDV, REV and CIAV (316, 677, 678, 713). Unfortunately, MDV stocks occasionally have become contaminated with other viruses, especially when propagated in chickens. Problems with CIAV contamination invariably interfere with the evaluation of MDV stocks for relative virulence (418).

Pathology

Gross Pathology

Pathologic changes in MD have been reviewed (482, 483, 485) and consist mainly of nerve lesions and visceral lymphomas. Enlarged peripheral nerves are a frequent finding in affected birds. Macroscopic changes are not seen in the brain, but gross enlargements can be found in spinal ganglia. Lesion distribution appears to be similar for naturally occurring and experimental diseases (471, 484). Goodchild (233) found that many nerves and plexi were commonly affected, but the celiac plexus was most commonly involved. Usually, plexi of the sciatic and brachial nerves are more enlarged than the respective trunks. Witter (697) has found the cervical vagus to be of particular diagnostic importance.

Nerves

Severely affected peripheral nerves may show loss of cross-striations, gray or yellow discoloration, and sometimes an edematous appearance. Localized or diffuse enlargement causes the affected portion to be 2–3 times normal size, in some cases much more. However, minimal enlargements may be important indicators of disease in experimental infections. Because lesions are often slight or unilateral, it is helpful to examine opposite nerves and, in experiments, to compare with age-matched normal controls to detect changes. Careful examination of the various nerve ramifications may be necessary to expose gross lesions in some birds, because enlargements can vary in both presence and degree from one portion of an affected nerve to another. Fig. 15.8 illustrates unilateral gross enlargements in the sciatic plexus.



15.8. Enlarged sciatic plexus (left) and normal plexus (right). (Peckham)

Visceral Organs

Lymphomas may occur in one or more of a variety of organs and tissues. Lymphomatous lesions can be found in the gonad (especially the ovary), lung, heart, mesentery, kidney, liver, spleen, bursa, thymus, adrenal gland, pancreas, proventriculus, intestine, iris, skeletal muscle, and skin. Probably no tissue or organ is without occasional involvement. Both the genetic strain of chicken and the virus strain can influence the organ distribution of lesions. Visceral lymphomas are common in more virulent forms of the disease (699). Visceral tumors can occur in the absence of gross nerve lesions, especially in certain strains of chickens. MD lymphomas in most viscera appear as diffuse enlargements, sometimes to several times the normal size, and a diffuse white or grayish discoloration is often present (Fig. 15.9B). Alternatively, lymphomas may occur as focal, nodular growths of varying size (Figs. 15.9E, F). Nodules are white or gray in color and are firm, and the cut surface is smooth. Necrosis is rare but may occur in the center of rapidly growing lesions.

Diffuse infiltration of the liver causes loss of normal lobule architecture and often gives the surface a coarse granular appearance. Nodular tumors may also be seen in the liver. Lesions in the immature ovary are observed as small to large grayish translucent areas (Fig. 15.9B). With large tumors, the normal foliated appearance of the ovary is obliterated. Mature ovaries may retain function, even though some follicles are tumorous. Marked involvement is indicated by a cauliflower-like appearance. The proventriculus becomes thickened and firm as a result of focal leukotic areas within and between the glands, which may be seen

through the serosal surface or, if involvement is diffuse, detected by palpation. Affected hearts are pale from diffuse infiltration or have single or multiple nodular tumors in the myocardium (Fig. 15.9F), or pinpoint foci may be seen in the epicardium. Involvement of the lung (Fig. 15.9E) and proventriculus may be indicated by increased firmness of the organ upon palpation. Muscle lesions may be present in both superficial and deep layers and are most common in the pectoral muscle (50). Gross changes vary from tiny whitish streaks to nodular tumors.

Integument

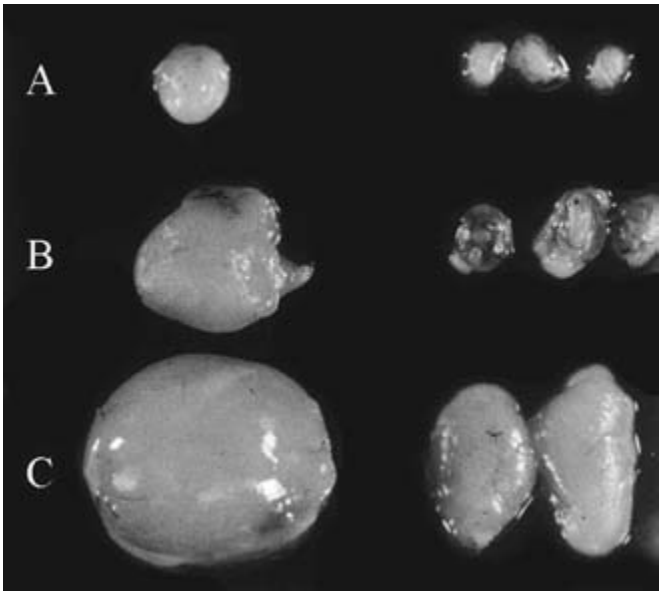
Skin lesions, probably the most important cause of condemnation in broiler chickens, usually are associated with feather follicles. The nodular lesions may involve few scattered follicles, or they may be numerous and coalesce. The distinct whitish nodules (Fig. 15.9A), especially evident in dressed carcasses, may become scablike with brownish crust formation in extreme cases (50). Lapen and Kenzy (362) found lesions in certain feather tracts more frequently than in others; the highest incidences were in external and internal crural and dorsal cervical tracts. Erythematous involvement of the shank integument is seen, especially in virulent forms of the disease in broiler chickens and is commonly known as "Alabama redleg." Swelling of the comb or wattles may indicate lymphoma growth in underlying tissues (199).

Eye

Gross ocular changes include loss of pigmentation in the iris ("gray eye") and irregularity of the pupil, both the result of mononuclear infiltration of the iris (Fig. 15.9C). Ficken *et al.* (214) described cases in which conjunctivitis, occasionally with multifocal hemorrhages and corneal edema, were observed. Witter (699) found that nearly all field isolates induced ocular lesions in nonvaccinated or HVT-vaccinated chickens; rates ranged from 5–100%.

Other Syndromes

Gross lesions are associated with at least some of the other syndromes that are associated with MDV infection. The lymphodegenerative syndromes, related to intense cytolytic infections of lymphoid organs, usually are characterized by severe atrophy of the bursa of Fabricius and thymus. The cytolytic infection is first evident 3–6 days after infection, but in some cases persists and is more obvious at 8–14 days after infection (Fig. 15.10) (113, 667, 721). After inoculation with highly virulent field strains, some chickens may die at 20–50 days without gross lesions except severe bursal and thymic atrophy (699). Some chickens also develop a transient splenomegaly within 4–12 days after inoculation (111). The splenomegaly is a nonneoplastic response to viral replication because it is induced by both virulent and avirulent serotype 1 strains as well as serotype 2 and 3 strains. Vascular syndromes are manifested principally by occlusive atherosclerosis (210). Susceptible P-line chickens inoculated with the CU2 isolate of MDV developed grossly visible fatty atheromatous lesions in large coronary arteries, aortas, major aortic branches, and other arteries (Fig. 15.9D). Lymphoid tumor transplants and local lesions are experimental syndromes characterized by nodular growths at the site of inoculation, although some transplantable tumors metastasize readily to the liver and spleen, causing diffuse enlargements (641). The gross appearance of transplants varies with the transplant, the host chicken, and the route of inoculation.



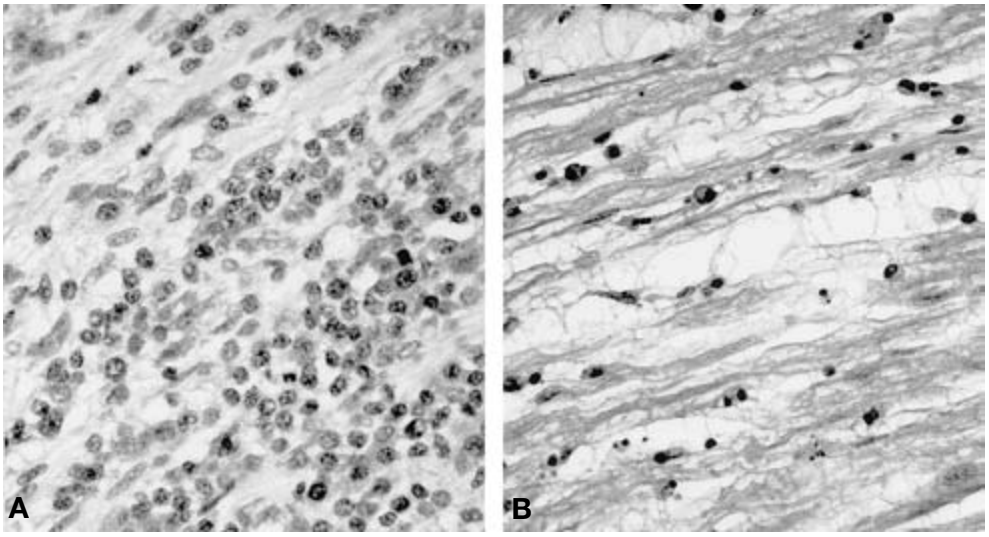
15.10. Atrophy of bursa (left) and thymic lobes (right) 15 days after inoculation of newly hatched chickens with A or B. A. The Md11 (vv) strain. B. The JM/102W (v) strain of Marek's disease virus. C. Age-matched uninoculated control tissues. (Courtesy of *Avian Diseases*) (Witter)

Microscopic Pathology

Histopathologic changes associated with MD lymphoproliferative lesions have been described by numerous workers who are in general agreement about the types of histologic lesions and the cells involved (480, 485).

Nerves

In peripheral nerves, 2 main types of lymphoproliferative lesions are recognized. One type is considered neoplastic, consisting of masses of pleomorphic lymphocytes; in some cases, demyelination and Schwann cell proliferation are associated with this lesion. The second type is essentially inflammatory and is characterized by diffuse, light-to-moderate infiltration by small lymphocytes and plasma cells, usually with edema, and sometimes, with demyelination and Schwann cell proliferation. A few macrophages may be found. Payne and Biggs (484) referred to these lesions as type A and B, respectively, and noted that the 2 types may be observed in different nerves of the same bird or even in different areas of the same nerve. Lawn and Payne (365) observed cellular infiltrations as early as 5 days PI, which gradually increased in intensity until 3 weeks when severe proliferative (type A) lesions were seen in the absence of paralysis or demyelination. Coincident with initial neurologic signs seen at 4 weeks PI, areas of widespread demyelination could be found within the

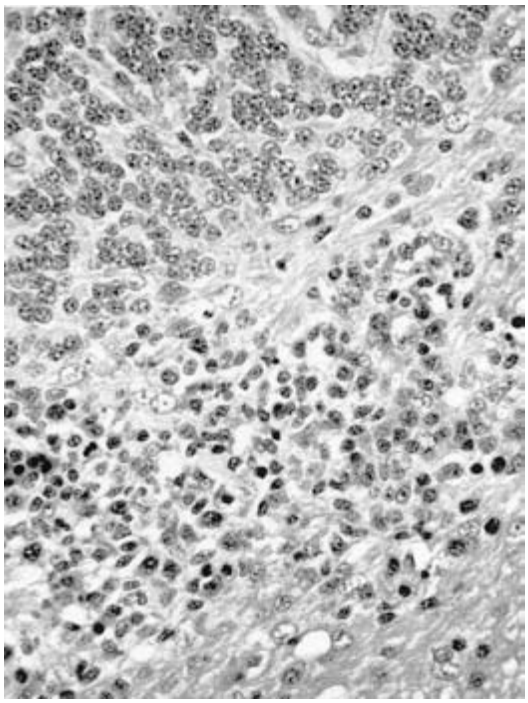


15.11. Microscopic lesions of Marek's disease in peripheral nerves. A. Type A lesion characterized by marked cellular infiltration, numerous proliferating lymphoblastic cells, and no edema. H & E, ×550. B. Type B lesion with edema, scattered infiltrating small and medium lymphocytes, and plasma cells. H & E, ×420. (Gimeno)

proliferative lesions. Finally, characteristic inflammatory (type B) lesions (edema, sparse infiltrations) appeared. The sequence of events has been reviewed in detail by Payne *et al*. (485). Characteristic changes in nerves are illustrated in Fig. 15.11.

Brain

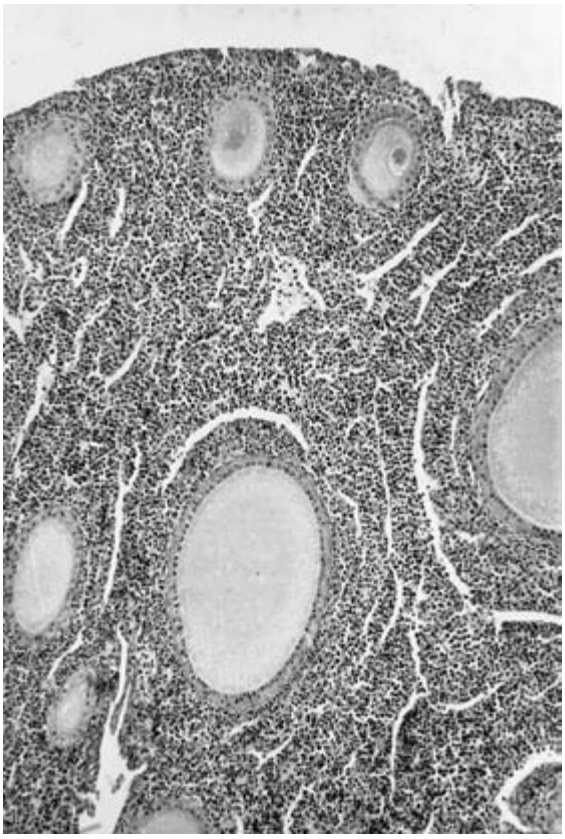
Initial reports identified mild perivascular cuffing usually accompanied by gliosis but without primary demyelination as the principal CNS lesion in MD (reviewed in 485). Wight (682) found that the CNS of affected birds was often histologically normal or with only minimal lesions. Following experimental inoculation with less virulent MDV strains, lesions could be seen as early as 7–10 days PI and were of moderate severity (488, 592). However, lesions induced by vvMDV strains appeared earlier and were more extensive (228). The initial lesions (also described under "CNS Syndromes") involve vascular elements; endotheliosis occurs at 6 days PI and is followed at 8–10 days PI by a moderate to severe infiltration of lymphocytes and macrophages around blood vessels and scattered throughout the neuropil (228). The vasculitis and edema disappear and may be followed by lymphoproliferative infiltrations of large lymphocytes and glial cells. These lesions tend to persist and are associated with persistent neurological disease. Severe lymphoblastic infiltration, often accompanied by extensive vacuolated areas that may correspond with secondary demyelination, has been shown to occur 4 weeks after inoculation with a highly virulent MDV (228). Infection with the C12/130 (a hypervirulent strain) and RK-1 (a vv+ strain) showed infiltration with monocytes in addition to T cells in the perivascular cuffs. These monocytes were expressing pp38 after infection with C12/130 (43) but not after infection with RK-1 (451). However in RK-1-infected birds many of the monocytes were in mitosis and apoptotic cells were often observed first in the white matter and later in the gray matter. Cho *et al.* (147) reported severe necrotizing and nonnecrotizing lesions in the brains of chickens up to 10 weeks PI with vvMDV. Thus, as in nerves, brain lesions are both inflammatory and lymphoproliferative. In contrast with nerves, however, the inflammatory lesions are induced first (231). Severe lymphoid infiltration in the cerebellum is shown in Fig. 15.12.



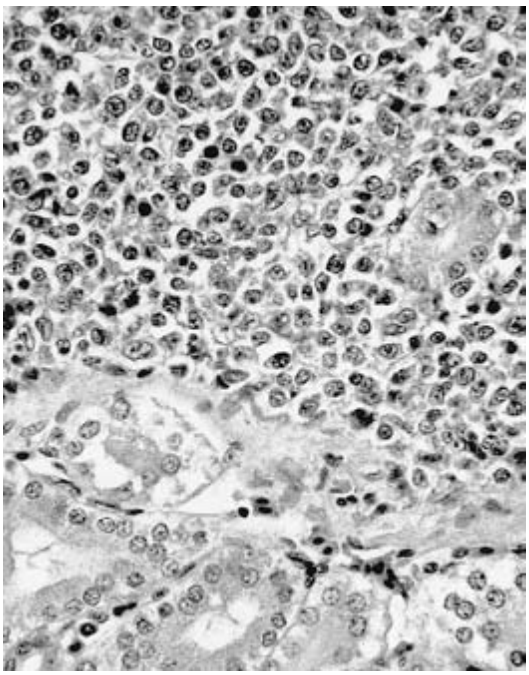
15.12. Extensive infiltration of lymphoblasts extending into the neuropil in the cerebellum of a Marek's disease virus-infected chicken 3 weeks PI. H & E, ×400. (Gimeno)

Visceral Organs

Lymphomatous lesions in visceral organs are more uniformly proliferative in nature than those in nerves (Fig. 15.13). Cellular composition is similar to that of the proliferative lesions de-



15.13. Lymphoid cell infiltration of ovary. Organ is composed largely of tumor cells, but a few ovarian follicles can be seen. H & E, ×116. (Witter)



15.14. Higher magnification of a kidney lymphoma showing pleomorphic tumor cells. Kidney tubules (bottom) show degeneration caused by tumor cell pressure. H & E, ×450 (Gimeno)

scribed for nerves, consisting of diffusely proliferating small-to-medium lymphocytes, lymphoblasts, and activated and primitive reticulum cells (527) (Fig. 15.14). Plasma cells are rarely present (527). Macrophages are also present in the tumor mass, especially in slow-growing tumors, perhaps reflecting the host immune response (52). The cellular composition of tumors is similar from one organ to another, even though the gross pattern of involvement may vary. Ultrastructural features of tumor cells have been described by several workers (191, 219). Pradhan *et al.* (515) found immune complexes in the kidney, leading to glomerulopathy, in MDV-infected chickens. They suggested that these lesions might be one of the major causes of death in MD.

Integument

Lesions in the skin appear largely inflammatory but may also be lymphomatous. They usually are localized around infected feather follicles. In addition, compact aggregates of proliferating cells, often perivascular, and a few plasma cells and histiocytes may be seen in the dermis (255, 484). With small lesions, the architectural integrity of skin is maintained, but massive proliferative lesions may cause disruption of the epidermis, resulting in an ulcer. Moriguchi *et al.* (427) described both inflammatory and lymphoproliferative lesions in the feather pulp; the latter were closely related to the incidence of MD. Feather pulp lesions may be useful for antemortem diagnosis. Lymphoproliferative nodules often surround feather follicles that contain MDV viral antigens and intranuclear inclusions in the FFE (110, 149).

Eye

The most constant change in the eye is mononuclear infiltration of the iris, but infiltrates may also be found in eye muscles, especially in rectus lateralis and ciliaris (324). Granular or amorphous material is sometimes present in the anterior chamber. Other, but more rarely, observed lesions involve the cornea (near Schlemm's canal), bulbar conjunctiva, pecten, and optic nerve. The unusually severe ocular lesions described by Ficken *et al.* (214) include uveal changes with increased aqueous humor protein and vascular engorgement and mild hyperemia to severe swelling of the iris. These authors also observed severe inflammatory changes and edema of the cornea, including intranuclear inclusion bodies. Sevoian and Chamberlain (591) and Smith *et al.* (629) reproduced ocular lesions experimentally. The latter reported that infiltration of proliferating lymphoreticular cells in optic and ciliary nerves and uvea were followed by similar infiltrations throughout the eye. Dukes and Pettit (193) found cataracts present in 7 of 18 spontaneous cases of ocular MD.

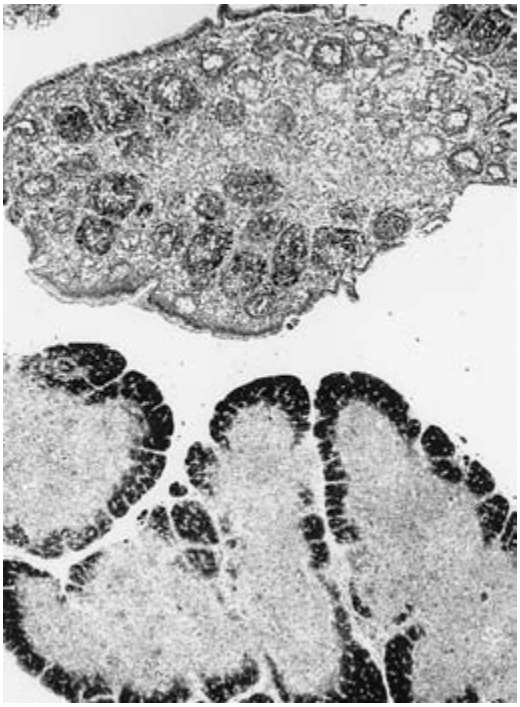
Blood

Blood leukocyte counts may be elevated, largely because of increased numbers of large lymphocytes and lymphoblasts (477). Payne *et al*. (487) identified the majority of leukemic cells as T cells. The leukemic response is not consistent and may be absent, or only a mild leukocytosis may be present (324, 592). Infection

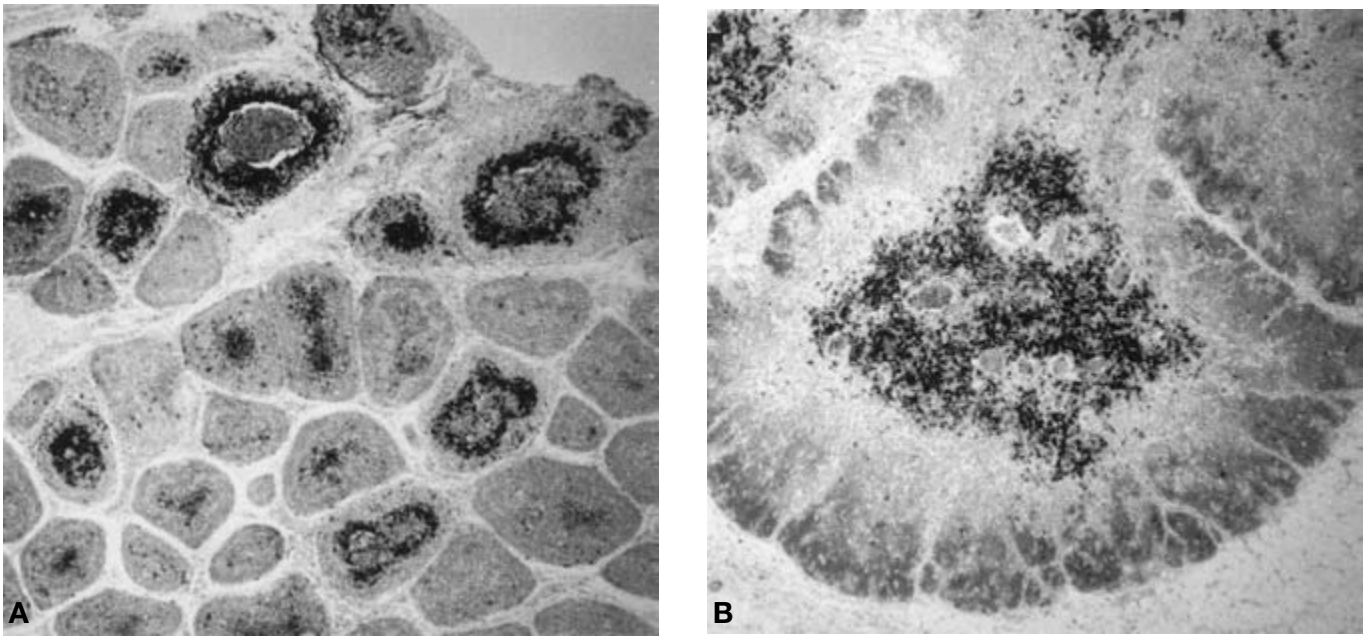
with the hypervirulent C12/130 strain caused significant increases in the absolute number of blood monocytes around 8 days PI. B cells, $CD4^+$ and $CD8^+$ T cells decreased during the early cytolytic infection followed by an increase between 8 and 10 days PI, but these changes were also seen for T cells after infection with HPRS-16 (43). Bone marrow changes in MD have variously been reported to include multiple tumor nodules (592), aplasia (309) but the MDV strain was later found to be contaminated with CIAV (567), or changes were not observed (527). Gilka and Spencer (226) described an extravascular hemolytic anemia in MDV-inoculated chickens characterized by reduced packed cell volumes. The importance of this finding is not clear because hematocrit values are not routinely used as a MD parameter.

Lymphodegenerative Syndromes

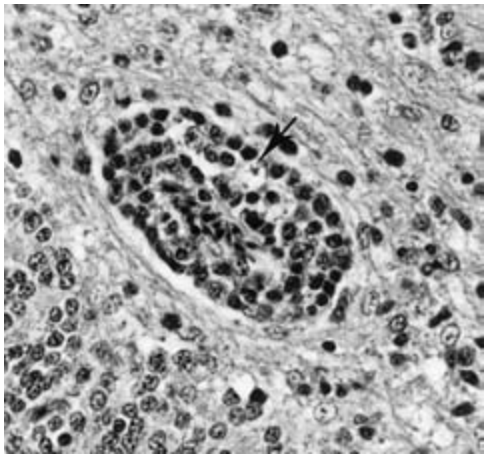
Productive herpesvirus replication in the bursa of Fabricius and thymus results in transient, acute cytolytic changes in these organs accompanied by atrophy (citations in 105, 485). In experimental infections, bursal lesions consist of follicular degeneration, lymphoid necrosis with depletion, and cyst formation (Fig. 15.15 *top*). Thymic atrophy is often severe, and lymphocytes are depleted in both cortex and medulla (see Fig. 15.15 *bottom*). Intranuclear inclusions can sometimes be found in cells associated with degenerative lesions. Viral antigens can be abundant during the acute cytolytic phase, especially in the medullary regions of the thymus and in some but not all bursal follicles (see Fig. 15.16). Chicks infected in the absence of maternal antibody may develop focal or generalized necrosis in a variety of organs, including the kidney (100, 215, 309). Following the acute cytolytic phase, antigen-positive cells disappear, and at least partial repopulation with lymphocytes occurs. Bursal and thymic atrophy, however, may persist for several weeks or longer. In the



15.15. Degenerative lesions in bursa and thymus of chickens inoculated with the 648A (vv+) strain of Marek's disease virus. Bursa (top) at 10 days PI shows degeneration and atrophy of follicles. Thymus (bottom) at 6 days PI shows necrosis and lymphoid cell depletion. ×12 (Gimeno)



15.16. Acute cytolytic infection of lymphoid tissues 6 days PI with the 648A (vv+) strain of Marek's disease virus. The pp38 viral antigen is visualized by immunohistochemical staining (black). A. Bursa of Fabricius. B. Thymus. ×30 (700) (Witter)



15.17. Transient paralysis lesions in the brain. Vasculitis in the cerebellum at 10 days PI showing endothelial cell necrosis, lymphoid cell accumulations, and vacuolization. Note intramural necrotic debris (arrow) as well as infiltration of heterophils in the vessel wall. H & E, ×250. (Gimeno)

bursa, some interfollicular lymphoid infiltration with T cells may occur.

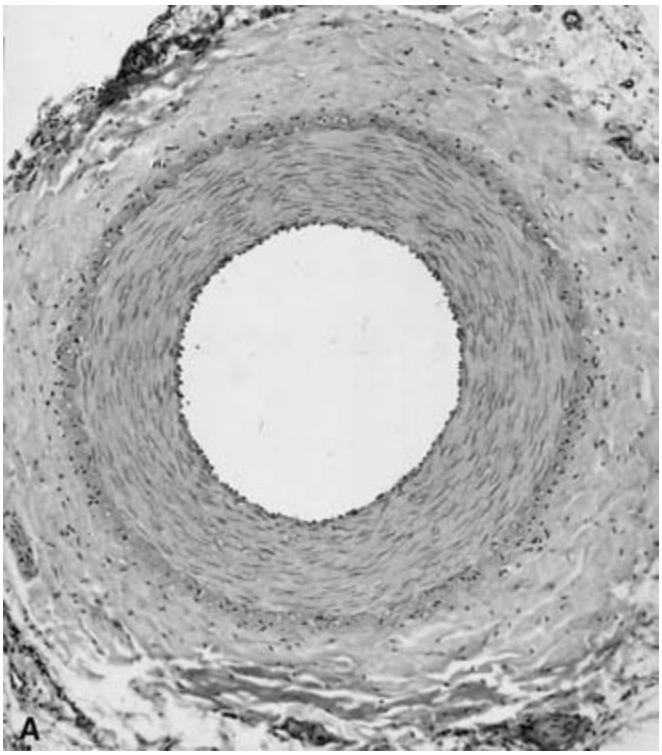
The early mortality syndrome, although normally characterized by severe lymphoid degeneration and death, often with enlarged, necrotic spleens (721), has recently been linked with central nervous system signs and lesions associated with transient paralysis (710).

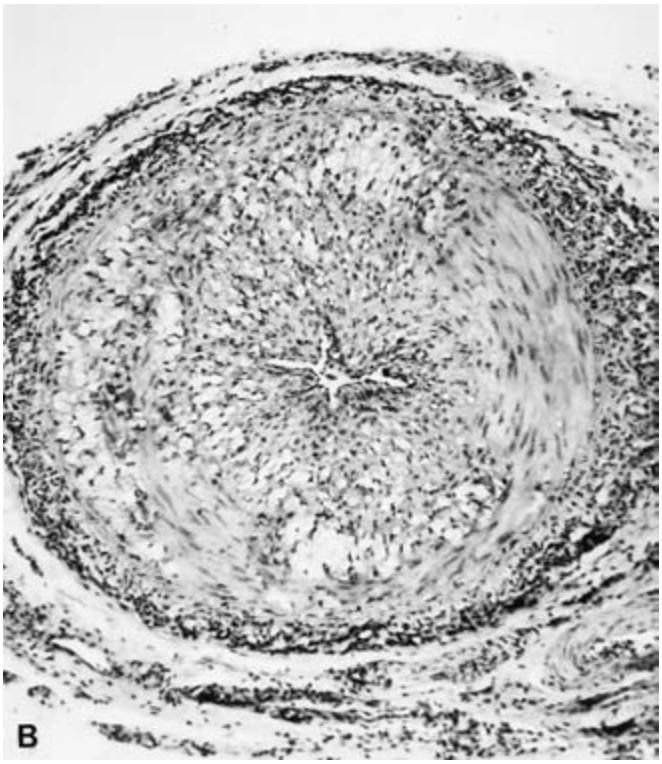
CNS Syndromes (Transient Paralysis)

Swayne *et al.* (644, 645, 646) reported that the critical lesion in transient paralysis was vasculitis (Fig. 15.17), which resulted in vasogenic brain edema. Leakage of immunoglobulin G (IgG) and albumin around affected vessels resulted in vacuolization. The edema and vasculitis were expressed coordinately with clinical flaccid paralysis and resolved in 2–3 days. Other, apparently unrelated, brain lesions (perivascular cuffing, lymphocytosis, and gliosis) could be observed after clinical recovery or in infected but clinically normal birds. Ultrastructural changes did not include demyelination (355, 645). CNS pathology associated with an acute (lethal) form of transient paralysis was similar in nature, albeit somewhat more severe, than in the classical syndrome (231, 710).

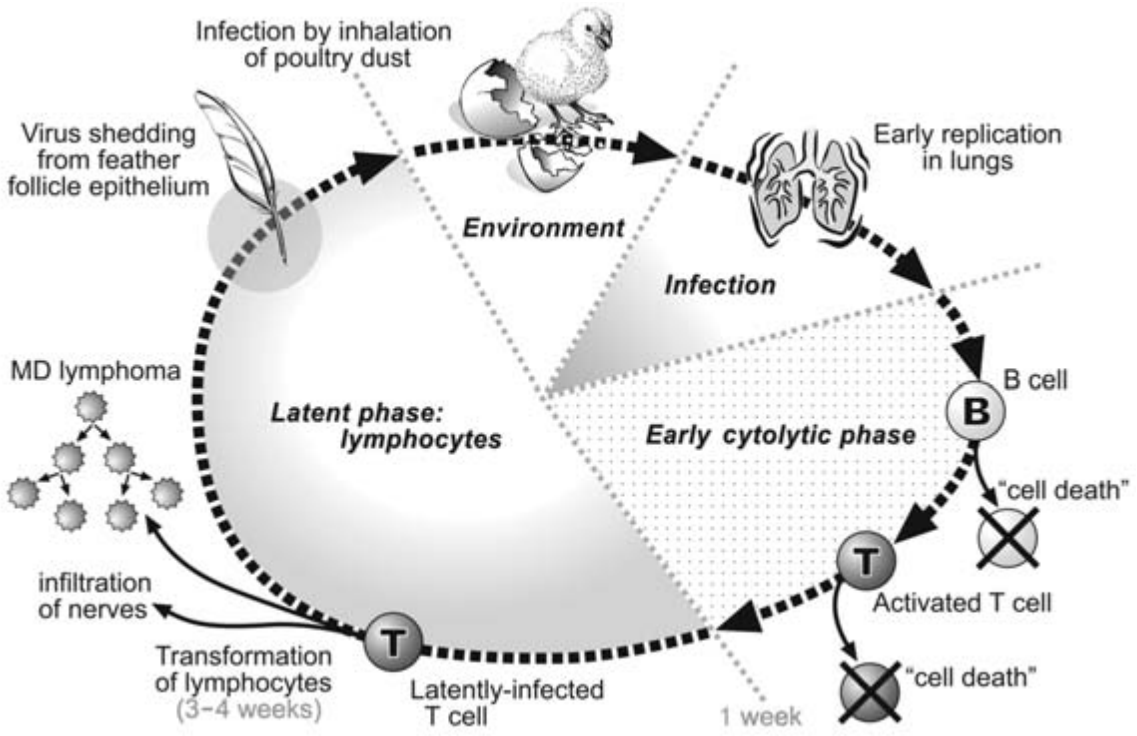
Vascular Syndromes

Arterial lesions reported to be associated with MDV-induced atherosclerosis include proliferative and fatty-proliferative changes in aortic, coronary, celiac, gastric, and mesenteric arteries (210, 419) (Fig. 15.18A, B). Internal and medial foam cells, extracellular lipid, cholesterol clefts, and calcium deposits characterized the fatty-proliferative lesions. Also, MD viral antigens could be detected by immunofluorescence adjacent to the arterial lesions. An altered lipid metabolism is suggested by the finding of Fabricant *et al.* (211) that MDV infection of arterial smooth muscle cells *in vitro* induced accumulation of phospholipids, free





15.18. A. Gastenic artery of normal chicken. B. Atherosclerotic artery in gizzard of chicken infected with CU2 isolate of Marek's disease virus. Lumen is occluded by thickened intima, and atheromatous changes have occurred deep in the intima and media. H & E, ×24. (C. Fabricant)



15.19. Schematic diagram showing the different stages of MD pathogenesis including the virus shedding from the feather follicle epithelium and the transformation of T lymphocytes in susceptible birds.

fatty acid, cholesterol, and cholesterol esters. *In vivo* studies supported this conclusion; Hajjar *et al.* (243) found that lipid accumulations in aortas resulted, in part, from altered cholesterol or cholesteryl ester metabolism during early stages of the disease.

Tumor Transplants and Local Lesions

Tumor transplants are composed of uniform, lymphoblastic cells with few, if any, infiltrating host cells (641) or, particularly in regressing tumors, may include small lymphocytes, heterophils, vascular invasion, and necrosis (216). Local lesions induced in the wing web or pectoral muscle by inoculation of MDV-infected, allogeneic kidney cells are inflammatory in nature, consisting of lymphocytes and macrophages and sometimes accompanied by hemorrhage and necrosis (118).

Pathogenesis

Several recent reviews on the pathogenesis of MD have been published (32, 107, 584). The review by Calnek (107) also provides several references for older reviews. The use of BACs and overlapping cosmid technologies has allowed the deletion of specific genes. Many of the deletion mutants have been examined in cell culture (467), but the importance of only a few genes has been established for the pathogenesis of MDV. These will be discussed in the appropriate sections of the pathogenesis. Unfortunately, the oncogenic RB-1B BAC clone (501) does not spread horizontally (313), which limits the information on specific genes for the replication in the FFE.

Four phases of infection *in vivo* can be delineated: 1) early productive-restrictive virus infection causing primarily degenerative changes; 2) latent infection; 3) a second phase of cytolytic, productive-restrictive infection coincident with permanent immunosuppression; and 4) a proliferative phase involving nonproductively infected lymphoid cells that may or may not progress to the point of lymphoma formation (Fig. 15.19). This division is somewhat arbitrary and phases 2–4 can coexist in different cells in the same bird. Infection with some of the vv+ strains may not follow this general pattern, and mortality can occur without even entering into the latent phase. The next section, however, will describe the classical pathogenesis in lymphoid tissues, based mostly on studies in SPF chickens. The pathogenesis of infection in the FFE involves epithelial cells and will be discussed in the section on cytolytic infection in FEE.

Early Productive-restrictive Infection (Phase 1)

The virus enters the host via the respiratory tract and cell-free virus reaches the lymphoid organs within 24–36 hours after intratracheal inoculation (562). MDV is probably transferred to the lymphoid organs by phagocytic cells, presumably macrophages. Recently Barrow *et al.* (42) provided support for this hypothesis by demonstrating the presence of MDV transcripts in the cytoplasma and nucleus of splenic macrophages. Because virus particles could not be demonstrated, it is not sure if this is an abortive or productive infection. Shortly thereafter, cytolytic infection can be detected in the spleen, bursa of Fabricius, and thymus, peaking at 3–6 days. Shek *et al.* (612) discovered that the primary target cells in all 3 organs are B cells. Activated but not

resting T cells can also undergo cytolytic infection (122, 124, 125). Baigent *et al.* (33, 36, 37, 42) confirmed that the early cytolytic infection occurs mostly in B cells using dual staining techniques with MAb specific for B and T cell markers and a pp38-specific MAb. In addition, they demonstrated that a small percentage of $CD4^+$ and $CD8^+$ T cells expressing TCRαß1, TCRαß2, and TCRγδ can become cytolytically infected during the early phase of the pathogenesis. The consequence is a transient atrophy of the lymphoid organs, especially the thymus and the bursa. Depending on the virulence of the challenge strain, birds may recover between 8–14 days PI, or the atrophy may become permanent (111, 113). The cytolysis is likely initiated by the activation of the host shut-off protein (see "Virus Replication") leading to cell death by apoptosis (429). Although MDV-infected cells in the thymus are mostly B cells (612), thymocytes undergo massive apoptosis possibly as the consequence of viral infection (36) or virus-induced cytokine changes.

Changes in proinflammatory cytokine expression occur in splenocytes during the cytolytic phase. The level of upregulation and the cytokines involved may depend on interactions between the pathotype of the virus and the genotype of the host (311, 325). Several groups reported the upregulation of IFN-γ mRNA in splenocytes as early as 3 to 4 days PI (190, 311, 315, 325, 733), but not in circulating blood leukocytes (534). Upregulation of IL-1ß and IL-8 has been reported (311, 733) but not by others (325). Two other proinflammatory cytokines, IL-6 (311, 325) and IL-18, are also upregulated (325). In addition to the cytokines, inducible NO synthase (iNOS, officially named NOSII) is also upregulated during the cytolytic infection (733) (see "Immunity").

The increased expression of cytokines may also explain the hyperplasia of lymphoid and reticulum cells (485), causing splenomegaly (111). The level of infection is in general similar in genetically resistant and susceptible strains during the early cytolytic period (4, 213, 325, 740). However, genetically resistant line 6 chickens have a significantly lower level of infected lymphocytes than susceptible line 7 chickens. Lee *et al.* (372) suggested that this was due, in part, to a deficiency in the aggregate number of target cells in line 6. Baigent *et al.* (33, 36) showed that line 7 birds have more cells expressing pp38 during the cytolytic phase than line 6 birds, but that there are more B cells in the spleens of line 6 than line 7 chickens. In spleens of line 7 birds, dramatic changes occur with irregular patches of $pp38^+$ B cells becoming surrounded by $TCR\alpha ß1^+$ $CD4^+$ and $CD8^+$ cells, thus providing optimal conditions for virus transfer from B to T cells. These data suggest that MDV may replicate and spread more efficiently in line 7 than line 6 chickens. Interestingly, Calnek *et al.* (124) reported that B cells from line 7 are more susceptible to *in vitro* infection than B cells from line 6.

The early activation of IFN-γ may be important for the upregulation of the IL-8 receptor on activated T cells, so that vIL-8 can attract activated T cells to lytically infected B cells and facilitate the transfer of virus to T cells (475, 584). Recent studies using deletion mutants lacking vIL-8 or the first exon of vIL-8 clearly support the importance of vIL-8 for the early cytolytic infection. Infection with these mutants resulted in a significant reduction in virus replication during the early lytic infection and a subsequent decrease in tumor incidence (163, 168, 314, 475). In addition to vIL-8, pp38 is also essential for the early cytolytic infection (229). Deletion of *meq*, RLORF4, RLORF5a, vLIP (LORF2), or viral telomerase did not significantly impact phase 1.

Several factors can modify the early pathogenesis. Prior vaccination and the presence of maternal antibodies reduce the cytolytic infection (100, 121, 574, 628). The reduction in cytolytic infection also will reduce the number of latently infected cells and reduce or delay tumor development. Exposure at 1 day of age prolongs the cytolytic infection compared to exposure at 2 or 7 weeks of age (97). Likewise, the pathogenicity of the virus strain may affect the severity of early infection. The vv strains (e.g., Md5) and vv+ strains (e.g., RK-1) can cause more severe lymphoid organ atrophy than the less oncogenic strains, resulting in an early mortality syndrome (113, 721).

The apoptosis of lymphocytes during the early cytolytic phase may cause transient or permanent immunosuppression, depending on the virulence of the challenge strain. In addition, a transient suppression of mitogen stimulation has been reported, but this may actually represent a protective response (581, 584). The importance of these observations is discussed in "Immunosuppression."

Latent Infection (Phase 2)

At about 6–7 days, the infection becomes latent when cytolytic infection can no longer be demonstrated, and tumors are not yet detectable. The development of latency coincides with the development of immune responses. The interactions between virus and cells during the induction of latency are incompletely understood. Impairment of cell-mediated immunity (CMI) delays the start of latency (97). The onset of latency is also delayed after infection with the more virulent pathotypes (740). Several soluble factors have been implicated in the induction of latency including IFN-α, IFN-γ, latency maintaining factor or LMF, and NO (96, 671, 734). Based on infection of CEF with RB-1B in the presence of IFN-containing supernatants, Levy *et al.* (381) suggested that IFN may block virus replication before translation of late genes.

Most latently infected cells are activated $CD4^+$ T cells, although $CD8^+$ T cells and B cells can also be involved (125, 376, 557, 612). Infection in genetically resistant birds often remains latent and can last for the lifetime of the bird (729), aside from a persistent low-grade productive infection in the FFE (101, 114, 372, 636). Apoptosis of T cells during latent infection has been described (428, 430), although it cannot be excluded that MDV was reactivated in these cells. Susceptible birds or resistant birds infected with vv or vv+ strains may develop a second wave of cytolytic infections after the second or third week, coincident with permanent immunosuppression.

The extent to which nonlymphoid cells are latently infected is not known, although apparent latent infection has been observed in Schwann cells and satellite cells in spinal ganglia (499).

Second Phase of Cytolytic Infection (Phase 3)

The second cytolytic infection phase has not been studied in great detail. It occurs in lymphoid organs, and localized foci of

infection can be found in tissues of epithelial origin in various visceral organs (e.g., kidney, pancreas, adrenal gland, proventriculus, etc.). Focal cell death and inflammatory reactions develop around affected areas (9, 107). The secondary cytolytic infection does not always occur. The development and extent of the secondary cytolytic infection depends on genetic resistance of the host and the virulence of the MDV strain.

Cytolytic Infection in FFE

Cytolytic infection occurs also in the FFE (114). The replication of virus in the FFE is unique in that it is the only known site of complete virus replication. The replication occurs in genetically resistant as well as susceptible birds independently of the virulence of the MDV strain. MDV most likely is transferred to the FFE by infected lymphocytes. Viral DNA of CVI988 was detected as early as 7 days PI by qPCR (38), although it is not known if this represents infectious cell-free virus. Lymphocyte aggregates consisting of small lymphocytes with nuclear inclusions can be detected in the perifollicular dermis as early as 7 days PI (148). The lymphoid aggregates can develop into either necrotic areas consisting of FFE cells and degenerating lymphocytes or into cutaneous tumors. The former is associated with strong expression of pp38, but the latter has only a few $pp38^+$ cells. It is likely that virus is reactivated from latency in the FFE, because mutant strains lacking vIL-8 (168) or pp38 (229) are able to produce virus in the FFE. Niikura *et al.* (450) examined the time course of protein expression in the FFE. pp38 is the first protein expressed in lymphocytes after reactivation, followed by the expression of gB in the inner layers of the epithelial cells. Finally, gD is expressed in the cell layer in contact with the feather shaft. The importance of expression of gD is unclear because gD deletion mutants do spread like wild-type virus (18).

Development of Lymphomas (Phase 4)

Lymphoproliferative changes, constituting the ultimate response in the disease, may progress to tumor development. Death from lymphomas may occur at any time from about 3 weeks onward. Regression of lesions has been reported after infection with vMDV strains and depends on the genetic resistance of the bird and the age at infection (89, 608).

The composition of lymphomas is complex, consisting of a mixture of neoplastic, inflammatory, and immunologically committed and noncommitted cells (490). Both T and B cells are present, although the former predominate (489). The transformed T cells are mostly $CD4^+$ cells expressing TCRαß1 or TCRαß2 and MHC class II (577). Infection of chickens in the wing web or breast muscle (118) or of chickens depleted of T cells (458) showed that other subsets (e.g., $CD8^+$ $CD4^-$, $CD3^-CD4^-CD8^-$ and $CD3^+CD4^-CD8^-$) can be transformed (458, 577). Burgess *et al.* (91) further characterized tumor cells using *ex vivo* lymphoma cells and tumor cell lines as MHC class I^{hi}, MHC class II^{hi}, $CD4^+$, $TCR\alpha ß1^+$ or TCRαß2, $CD28^-$, $CD30^{hi}$, $IL\text{-}2R\alpha^+$. The expression of high levels of CD30 using MAb AV37 led Burgess *et al.* (93) to suggest that MD could be a natural model for Hodgkin's disease. In addition to these markers, tumor cells may also express MATSA (731) and various poorly characterized fetal antigens (508).

The infection in transformed cells is nonproductive *in vivo* and *in vitro*. $CD4^+$ and $CD30^{hi}$ tumor cells express Meq and SAR (see "Viral Genes and Proteins"), but are negative for pp38 and gB (91, 557). Additional genes, for example RLORF4 (312) and RLORF5a (456), may also be expressed in tumor cells, but the importance of their expression remains unknown. Low numbers of tumor cells may express pp38, which probably represents cells in which a productive-restrictive infection has been initiated. Based on *in vitro* studies it is suggested that the expression of pp38 and late genes in tumor cells may be under control of IFN (381, 672).

The possibility that MD tumors are of clonal origin has been proposed based on observations of random MDV DNA integration into the genomes of lymphoma cells (187). Although integration was random, the pattern of integration sites among cells from a given lymphoma or a given lymphoma-derived cell line was consistent. This work has fundamental implications regarding the pathogenesis of MD but awaits confirmation and further definition. Earlier studies by Schat *et al.* (577) showed that different lymphomas in the same bird could yield cell lines representing different T-cell phenotypes.

Studies of graft-versus-host reactions in different genetic strains led to the suggestion that low alloimmune competence and resistance to MD are very closely related through genetic linkage or functional dependence (398, 491). This prompted speculation by Schat *et al.* (578) and Calnek (105) that the activation of T cells in response to the lytic infection of B cells constitutes a significant event in the pathogenesis of MD by providing an abundant supply of cells that are the usual target cells for transformation. This hypothesis has been borne out by studies by Calnek *et al.* (108, 118) showing that tumor induction at the site of inoculation with MDV is enhanced by provoking a CMI reaction against allogeneic cells at the site. It is plausible that transformation requires 1) susceptibility to infection; 2) intrinsic or extrinsic control of virus replication (latency); 3) cell division to integrate virus genome; and 4) expression of viral oncogenes, activation of cellular oncogenes, or repression of the induction of apoptosis. Activated T cells infected at the time that cytokines and/or CMI responses cause a switch to latency could fit this model. Interestingly, cells present as early as 4 days after inoculation of MDV-infected allogeneic CKC can be grown *in vitro* as MD cell lines (118). Thus, transformed cells, or at least transformation target cells, may be present even during the early cytolytic phase of MD.

Factors Influencing Pathogenesis

The pathogenesis of infection with oncogenic MDV, which has become attenuated by passage *in vitro*, has been studied by Bradley *et al.* (quoted in 485) and Schat *et al.* (576). Both groups found that attenuated virus failed to cause cytolytic infection of lymphoid organs and that cell-associated viremia levels were low. The latter authors further learned that attenuated virus was not infectious for lymphocytes *in vitro*, perhaps explaining the *in vivo* observations.

The actual mechanism(s) by which pathogenesis is altered in the case of host resistance is not clear. CMI probably is involved,

however, and evidence (see "Immunity") suggests that immune responses of the host may be directed against either the early virologic events or the later proliferative phase and that an effective response at either stage might reduce the chance of overt disease. Both age and genetic resistance are dependent on immunologic competence (101, 610). The availability of appropriate target cells for viral replication is also important as was demonstrated for line 6 versus line 7 (33, 372). If the hypothesis is correct that tumor development is enhanced by a strong T-cell response against the early cytolytic infection of B cells, then factors that limit that response should reduce tumor incidence. Vaccinal immunity, embryonal bursectomy, and splenectomy suppress the active viral infection (121, 563, 573, 574, 628), thereby obviating an inflammatory response and reducing the incidence of tumors. Interestingly, evidence (citations in 103, 108, 117) shows that some genetic strains with unusually strong CMI responses are especially susceptible to MD, although this is not true in all cases.

Virus strains differ in oncogenicity, but the molecular basis for differences in pathogenesis associated with the strains are not well defined. All cause similar early cytolytic infections, although the vv+ strains may cause a prolonged and more severe cytolytic infection. Some of the new strains are capable of infecting macrophages leading to increased death of macrophages (37, 42, 44).

The immune response itself may be responsible for some lesions characteristic of MD. Nerve lesions have some characteristics suggestive of an autoimmune disease (523, 586), and MD has been identified as a model for the Landry-Guillain-Barré syndrome (499). Additional evidence supporting an autoimmune component for MD comes from studies showing immune complexes in the kidneys of MDV-infected chickens and quail (336, 515).

As indicated previously, these events are based on studies in SPF chickens under controlled conditions. However, the primary cytolytic infection is not an absolute prerequisite for tumor development. Schat *et al.* (573) found that MDV infection in embryonally bursectomized chickens resulted in the development of tumors in the absence of the primary cytolytic infection. These results are similar to the development of tumors in the absence of cytolytic infection during phase 1 of the pathogenesis after infection using vIL-8 and pp38 deletion mutants (168, 229, 314, 475). Apparently, enough activated T cells are available at the time of challenge to become infected. Thus, cytolytic infections may not be absolutely essential for the development of tumors, which also may be the case in vaccinated, commercial chickens. However, stress and immunosuppressive infections perhaps may induce secondary cytolytic infections, reducing the benefits provided by vaccination.

Pathogenesis of in ovo Infection

An understanding of the pathogenesis of infection after *in ovo* injection of the 3 serotypes has become important in view of the widespread use of *in ovo* vaccination. HVT could be isolated from embryonal lung tissues at 1 day PI in the amniotic fluid at ED 17, while chorioallantoic membranes, embryonal spleen, and proventriculus remained negative. However, spleens were virus positive at 4 days post hatch (605). Studies using *in situ* hybridization confirmed these findings (640).

The target cells for HVT in the lung are adherent fibroblastoid or epitheloid cells, but few cells underwent a lytic infection based on the absence of viral proteins (599) and the lack of apoptosis (639). It is possible that HVT did not replicate and that the high titers (605) are the consequence of rapid cell division of infected cells during embryonal development. Perhaps the expression of the anti-apoptotic ORF in HVT (347) and/or the induction of high levels of IFN (600) are responsible for the lack of apoptosis and virus replication.

Low levels of SB-1 or attenuated serotype 1 strain Md11/75C could be recovered from embryonal lung tissues shortly before hatching after inoculation in the amniotic fluid at ED 16 to 18 (598). Intravenous inoculation of RB-1B, a vvMDV strain, at ED 16 resulted in active virus replication in the bursa of Fabricius at ED 21, while intra-amniotic inoculation resulted in the presence of viral DNA in lung and thymus tissue (598, 639, 640). Pathogenic strains and HVT, but not SB-1 or Md11/75C, induce high levels of IFN in embryos after inoculation in the amniotic fluid (600).

Pathogenesis of Nontumor Diseases

MDV infection can cause several nonneoplastic disease syndromes (Table 15.4B). The pathogenesis of MDV-induced atherosclerosis has not been elucidated. Microscopic lesions consisting of fatty proliferative lesions with alterations in lipid metabolism in arterial smooth muscle cells could be detected as early as 1 month after infection (208, 243). $CD4^+$ and $CD8^+$ T lymphocytes infiltrated into the intima as early as 2 weeks PI. In addition, endothelial cells expressed MHC class II antigen in infected but not in normal chickens (452). These cells may have been responsible for the introduction of virus into the smooth muscle cells leading to occasional viral antigen expression and alterations in the lipid metabolism. In contrast to the original work by Fabricant *et al.* (210, 211) Njenga and Dangler (453) were unable to demonstrate arterial lipid accumulation without supplementation of the feed with cholesterol. Intimal cellular infiltrates were detected, and serum cholesterol was increased significantly compared to noninfected control chickens.

The pathogenesis of the neurological lesion complex consisting of classical transient paralysis (TP), acute TP resulting in mortality within 1–3 days PI, persistent neurological syndrome (PND), and late paralysis (LP) (231) is not fully understood. The difference between classical and acute TP is somewhat arbitrary (710) and the early pathogenesis is probably similar. The development of both types of TP is influenced by the MHC and the virulence of the MDV strain with the more virulent strains causing acute rather than classical TP (585, 710). B cells are required for the induction of transient TP (476). The brain lesions start with vasculitis at 6–8 days followed by leakage of albumin from blood vessels into vacuoles (646). This vasogenic edema is transient and correlates with the clinical paralysis associated with classical TP (643). Jarosinski *et al.* (311) noted that the development of neurological symptoms induced by vv+ RK-1 correlated with increased levels of iNOS mRNA in the cerebellum and NO

in blood serum (See "Nitric Oxide"). NO can cause vasodilation and could be the cause of the edema. Chickens inoculated with the vMDV JM-16 strain did not show neurological signs or iNOS mRNA in the cerebellum.

The degree of virus replication in the brain may be related to the severity of the disease. Low levels of virus replication (3) or absence of virus replication (230) correlated with the absence of neurological symptoms. The importance of virus replication in the brain was also shown by comparing JM-16 and RK-1. The former had very low levels of virus replication in the cerebellum and did not induce symptoms, while the replication of RK-1 was significantly higher and produced lesions in MHC resistant and susceptible chickens (311). During attenuation of the vv+ strain 648A by serial passage in CEF, reduction in transient paralysis induction occurred coordinately with a reduction in the level of viral replication in lymphoid organs and FFE (230).

Endothelial cells expressed no viral antigens but became hyperplastic and hypertrophic soon after the virus reached the brain and showed upregulation of MHC class II antigen as early as 6 days PI and downregulation of MHC class I antigens at 10 days PI (228). Infection with RK-1 or RB-1B also caused upregulation of proinflammatory cytokine transcription in the cerebellum including IFN-γ, IL-1ß, IL-6, IL-8, IL-12, and IL-18 (3, 311). The increased expression of IFN-γ, IL-6 and IL-18 was significantly higher in birds with RB-1B-induced classical TP than in asymptomatic MDV-infected chickens (3).

The clinical signs of PND are associated with a strong infiltration of lymphoblasts in the neuropil, many of which express Meq protein, and the later occurrence of this syndrome (about 3 weeks PI) suggests that its pathogenesis may parallel that of lymphoma induction in other tissues. Moreover, persistent neurological disease was shown to be closely related with the onset of lymphoproliferative lesions in peripheral nerves and visceral organs (230).

Immunity

Infection with pathogenic MDV or vaccine strains not only results in the activation of innate or nonspecific and acquired or specific immune responses, but may also cause immunosuppressive effects especially after infection with pathogenic serotype 1 strains. The importance of the interactions between immune responses and immunosuppression for the pathogenesis of MD cannot be overemphasized. A distortion in the balance toward immunosuppression will lead to disease. Immune responses and immunosuppressive features of MD have been extensively reviewed (182, 431, 565, 581, 584).

Immune Responses

The immune responses developing during the early cytolytic phase of infection are crucial for the outcome of infection. Impairment of immune responses during this phase delays establishment of latency resulting in prolonging the lytic infection and the subsequent continued destruction of immune cells by virus-induced apoptosis. Impairments include infection at 1 day of age when the immune responses are not yet fully developed or treatment with cyclosporin or neonatal thymectomy combined with cyclophosphamide treatment (97). The importance of immune responses during latency is relevant for protection against the second cytolytic phase and is dependent on cell-mediated immunity. It has often been suggested that vaccine-induced immunity is an anti-tumor immune response because vaccination does not prevent superinfection with wild-type virus but does prevent tumor development. However, vaccination clearly reduces the early cytolytic infection (121, 574), thus preventing extensive damage to the immune system and reducing the number of latently infected T cells. Lesion regression, however, has been described (89, 608) suggesting that immune responses against tumor cells may occur. Burgess *et al.* (93) detected specific antibodies against CD30 in resistant line 6 but not in susceptible line 7 chickens after infection with the vMDV HPRS-16 strain. This important finding needs further study, because there is no evidence that HPRS-16 induced $CD30^{hi}$ cells (See "Development of Lymphomas") in line 6 birds (89, 93).

Initiation of Immune Responses. Professional antigen-processing cells (APC), such as dendritic cells, encountering pathogens are activated by interactions between the pathogen-derived pathogen-associated molecular patterns (PAMS) and the pattern recognition receptors (PRR), e.g., Toll-like receptors, on the APC. These interactions result in the activation of cytokines which direct both the innate and acquired immune responses. There is currently no information on PAMS associated with any of the 3 MDV serotypes.

Although the distinction between innate and acquired immune responses has become less defined over the last few years, the innate and acquired responses will be discussed in separate sections.

Innate Immune Responses. Innate immune responses include changes in cytokine expression, natural killer (NK) cells and macrophages.

Cytokine Responses. Infection with MDV results in the upregulation of a number of proinflammatory cytokines (See "Early Productive-restrictive Infection (Phase 1)" for details) driving a T_{H1} type of immune response. It is of interest to note that the vv+ RK-1 strain caused strong proinflammatory cytokine responses in spleen and cerebellum of MHC susceptible and resistant strains of chickens with significantly higher levels of IFN-γ, ILß-1 and IL-8 in the resistant line between 4 and 10 days PI without beneficial effects for the host (311). The authors speculated that a strong genetic-based response was actually detrimental for the host by inducing high levels of NO production.

IFN-γ is an important pleiotrophic cytokine with many functions in antiviral immune responses, but few studies have been performed on the roles of IFN-γ in protective immunity to MD. *In vitro* studies indicate that IFN-γ is able to inhibit virus replication directly or indirectly through the induction of NO production and reactive oxygen intermediates (188, 734).

The importance of IFN-α for MD immunity has not been analyzed in detail. Xing and Schat (733) failed to show induction of INF-α mRNA in spleens of control and infected resistant chickens. Quéré *et al.* (534) found a decrease of INF-α mRNA in

blood cells at 1 day PI in a resistant line of birds but not in a susceptible line. Stimulation of IFN-α mRNA by inoculation of infected chickens with NDV showed that MDV was able to block transcription at 1 day PI in susceptible but not in resistant birds. It will be of interest to determine if this result can be repeated in additional lines. Mixtures of IFNs containing IFN-α and -γ produced by stimulation of CEF with Newcastle disease virus were able to upregulate MHC class I expression in CEF and to counteract the decreased MHC class I expression induced by RB-1B (382).

Nitric Oxide. NO is synthesized by 3 isoforms of NOS with iNOS (NOS II) being inducible in macrophages, glial cells, astrocytes and perhaps other cells as well. The induction of iNOS occurs as part of the nonspecific immune response to microorganisms and is part of the inflammatory response. NO and other reactive nitrogen species are very versatile molecules with many functions independent of the source. NO has been linked to beneficial effects by killing pathogens, but also to neurodegenerative processes in humans (79, 194).

NO can inhibit MDV replication *in vitro* (188, 734). Increased transcription of iNOS has been reported between 6 and 12 days PI with MDV (733) resulting in increased levels of NO in the plasma of genetically resistant chickens but not in genetically susceptible chickens (190, 315). The production of NO may be beneficial, because it can inhibit MDV replication *in vivo* when genetically resistant chickens were challenged with vMDV (734). However, Jarosinski *et al*. (311) noted that pathology may be associated with very high levels of NO production especially in genetically resistant birds after challenge with vv+ MDV.

NK Cells. NK cells are the first line of defense because these cells can lyse virus-infected and tumor cells without prior exposure to the pathogen. NK cells are also potent inducers of IFN-γ. In order to lyse target cells, NK cells must recognize the target cells as foreign (e.g., the MHC class I has been altered, or the expression is downregulated). Sharma and Coulson (604) reported that NK cells are cytotoxic for MDCC-MSB1 cells, but Heller and Schat (253) found that most MDCC lines are resistant to NK cell lysis. The importance of NK cells for MD immunity has not been elucidated. Sharma (596) reported that tumor-bearing, genetically susceptible chickens had decreased NK cell activity in contrast to enhanced NK cell levels in tumor-free, genetically resistant or vaccinated chickens. NK-like activity was demonstrated in genetically resistant N2a chickens for at least 14 days after infection with RB-1B, while NK-like activity could not be demonstrated after 8 days in susceptible P2a chickens (224). NK cells are also activated early after vaccination with SB-1 and HVT (254). The enhanced NK cell activity could be beneficial when chicks become infected shortly after vaccination, perhaps by providing a source of IFN-γ or lysing virus-infected cells. The recently reported downregulation of MHC class I during lytic infection (281) certainly supports a potential role for NK cells.

Macrophages. Activated macrophages can restrict virus replication and reduce tumor incidence (238, 239, 261). Schat *et al.* (581, 584) suggested that these observations could be explained by the production of NO or by reactive oxygen intermediates (188). Macrophages harvested shortly after MDV infection can inhibit DNA synthesis and proliferation of MD lymphoblastoid cell lines *in vitro*, which was considered to be a transient immunosuppressive effect (373, 595). However, it is more likely that this inhibition is actually a protective response, because it limits the number of activated T cells during the critical switch of MDV from B to T cells (581, 584).

Humoral Immunity. Chickens infected with MDV develop precipitating and VN antibodies within 1–2 weeks; a transient immunoglobulin M (IgM) response is replaced by IgG (260). These antibodies are made against a large number of proteins (666). Most of these antibodies are not relevant for protective immune response, because they detect either nonstructural proteins or proteins that are not expressed in the virus envelope or on the surface of virus-infected cells. Due to the cell-associated nature of MDV antibodies are of limited importance in MD immunity. VN antibodies are important only when cell-free virus infects chickens or when MDV proteins are expressed on the surface of cells. In the latter case, antibodies plus complement or antibody-dependent, cell-mediated cytotoxicity can lyse infected cells. *In vivo* VN has indeed been demonstrated using cell-free and cell-associated virus (94). The presence of maternal antibodies reduces the cytolytic infection (100) and can reduce the efficacy of cell-associated vaccines with low titers or if cell-free HVT is used (e.g., 131, 346).

Few specific antigens involved in humoral immunity have been identified. Antibodies to purified gB neutralize cell-free MDV (292). Inoculation with recombinant fowlpox virus (rFPV) expressing gB (442) or inoculation with gB alone (463) resulted in the production of VN antibodies and protection against MDV challenge. Antibodies against other glycoproteins, such as gC, gE, and gI, are detected after infection. Inoculation with baculovirus-produced gC (310) or vaccination with rFPV expressing gC (442) did not protect against challenge.

The possibility that surface antigens found on MDV-transformed cells could be involved in immunity was raised by studies in which anti-idiotype antibodies against MATSA (see "Etiology") were shown to immunize chickens against challenge with virulent MDV (172).

A role for antibody-dependent cell-mediated cytotoxicity (ADCC) in MD immunity has been suggested (351, 549). However, the target antigens and effector cells have not been identified.

Acquired Immunity

Cell-Mediated Immunity. CTL (cytotoxic T lymphocytes) recognize small peptide fragments of 8–12 amino acids in the context of self-MHC class I antigens. These peptides are generated from *de novo* synthesized proteins through a complex process involving the proteasome and transporters associated with antigen-processing (TAP) 1 and 2. *In vitro* demonstration of antigen-specific CTL requires effector and target cells expressing the same MHC class I antigens (602).

Pratt *et al.* (521) stably transfected and expressed MDV genes in REV-transformed cell lines with known MHC antigens. These cell lines were used to show that CTL from infected or vaccinated chickens recognize peptides derived from pp38, Meq, ICP4, ICP27, gB, gC, gH, gI, and gE (409, 460, 584). The effector cells developed around 7 days PI and were characterized as typical CTL expressing CD3, CD8, and TCRαß1 but not CD4 (461). Important differences were noted in the recognition of proteins by CTL from resistant and susceptible chicken lines. CTL from resistant N2a (MHC: $B^{21}B^{21}$) but not from susceptible P2a (MHC: $B^{19}B^{19}$) chickens recognized ICP4 (460). Effective killing of infected cells as soon as ICP4 is expressed, e.g., when latently infected cells are reactivated, and before virus replication is completed could be one of the contributing factors to MHC-based genetic resistance. Two glycoproteins, gB and gI, were recognized by CTL from both lines. N2a-derived CTL also lysed cells expressing gC and gK and to a lesser degree gH, gL, and gM. CTL from P2a chickens recognized cells expressing gE. Chickens vaccinated with a rFPV vaccine expressing gB (rFPV-gB) developed CTL specific for gB (462) in addition to VN antibodies (442). Lee *et al.* (374) found that rFPV-gI, but not rFPV-gE or rFPV-gH, conferred protection to MDV challenge. It is likely that gB- and gI-specific CTL contribute to the protective immunity induced by recombinant vaccine. CTL against pp38 are likely to be important for immunity based on the observation that vaccination with rFPV-pp38 transiently reduced viremia levels in the absence of VN antibodies (442).

Vaccinal Immunity. HVT, attenuated MDV, and serotype 2 MDV protect against early replication of virulent viruses in the lymphoid organs of challenged birds and reduce the level of latent infection (121, 514, 574, 628). Based on current knowledge, the following sequence of events is proposed to explain vaccine-induced immunity with challenge occurring within 3 days after hatch as is typical in the field (See "Immunity" for references). NK cells are activated as early as 3 days post vaccination, probably producing IFN-γ and killing limited numbers of virus-infected B cells. IFN-γ is perhaps produced by other cells at this early stage (e.g., macrophages). IFN-γ can reduce virus replication and stimulate macrophages to initiate the transcription of iNOS, producing NO between 3 and 7 days post vaccination, thus limiting the replication of challenge virus. Antigen-specific CTL develop starting at 7 days post vaccination and may eliminate additional cells infected with challenge virus perhaps in combination with ADCC. The combination of these effector mechanisms will push the challenge virus into latency. Memory CTL will be able to quickly eliminate reactivated virus-infected cells.

Many factors can interfere with vaccine-induced immunity such as MDV-caused immunoevasion (see "Immunoevasion"). Concurrent infections with immunosuppressive viruses, e.g., CIAV (410) or stress may interfere with vaccine-induced cell-mediated immune responses. Deletion of humoral immunity by bursectomy and X-irradiation does not seem to have a major effect on protection conferred by attenuated MDV (203), although a similar treatment partially impairs vaccinal immunity from HVT (542).

Immunoevasion

Many viruses including MDV (568) have developed strategies of immunoevasion interfering with the development of immune response. Schat and Skinner (583) defined immunoevasion as "*Pathogen-initiated responses counteracting the immune responses to the specific pathogen". In vitro* infection with MDV causes downregulation of MHC class I (281, 340, 382) probably through retention of the class I molecule in the endoplasmic reticulum (281). Hunt *et al.* (281) suggested that HVT also downregulates MHC class I, but Levy *et al.* (382) were unable to confirm this. Transcripts for CD8α and ß chains are also downregulated during the cytolytic infection and at later stages resulting in a decreased expression of CD8 on T cells (428, 429) and perhaps NK cells. The production of INFs may upregulate expression of MHC class I to counteract the immunoevasive effects of infection (340, 382). The downregulation of CD28 on MD tumor cells (91) may interfere with antitumor responses. Other antigens, such as chicken fetal antigens, may interfere with NK cell activity (454).

Immunosuppression

Suppression of the immune response by MDV infection is a critical feature of the disease, contributing to the virulence of MDV isolates and altering susceptibility of the host to other pathogens (reviewed in 568). Initial impairment of the immune response is the results of the lytic infection of lymphocytes during the first cytolytic infection (see "Pathogenesis") (citations in 568). Permanent immunosuppression coincides with the second phase of cytolytic infection and tends to correlate with eventual tumor development (582) and may be seen only in birds that have already developed neoplasms (654). It is therefore difficult to distinguish between cause and effect especially because tumor cells might have suppressor activity (84, 253, 533, 653). Because immunocompetence is required for the maintenance of latency (97) it might be that immunosuppression associated with the appearance of transformed lymphoblasts results in additional reactivation of the lytic infection. This, in turn, will cause the loss of additional B and T cells, thus compounding the situation and resulting in the bursal and thymic atrophy seen in birds destined to succumb to MD. A possible association between immunosuppression, reactivation of cytolytic infection, and MD breaks during the laying cycle should be considered. However, immunosuppression may not be a prerequisite for the development of tumors. Witter *et al.* (716) found that the RM1 clone, derived from the JM strain of MDV by the insertion of a retroviral LTR following cocultivation with REV (318), was no longer oncogenic, but caused a severe early cytolytic infection. It will be important to further analyze the relationship between virus-induced immunosuppression and oncogenicity. Although the two properties are not invariably linked, they are often expressed concurrently and, in such cases, immunosuppression may serve to augment oncogenic potential.

Humoral and cell-mediated immunity can be suppressed by MDV infection leading to reduced antibody responses to a variety of antigens and alterations in T-cell functions, such as skin graft rejection, mitogen stimulation of lymphocytes, delayed hypersensitivity, reduced NK cell activity, primary and secondary

infections with coccidia, and impaired Rous sarcoma regression (citations in 485).

Diagnosis

Techniques for diagnosis of infection with MDV are different from those needed for differential diagnosis of the disease. The infection is ubiquitous, but the disease is not. The principal methods to identify the presence of infection are isolation of the virus, demonstration of viral DNA or antigens in tissues, and detection of antibody. The applications of different diagnostic procedures have been recently reviewed (457, 746).

Virus Isolation

Virus isolation is performed to confirm its presence for diagnostic purposes and to secure the infectious virus for further study. Techniques for isolation of all serotypes have been reviewed by Sharma (601).

Source of Virus

MDV can be isolated as early as 1 or 2 days PI (504) or 5 days after contact exposure (9) and throughout the life of the chicken. Intact viable cells are the preferred inoculum because, in most cases, infectivity is avidly cell-associated, although cell-free preparations from skin, dander, or feather tips of infected chickens may contain the virus (110). Inocula may consist of blood lymphocytes, heparinized whole blood, splenocytes, or tumor cells. The virus can often be recovered from infected cell suspensions following storage for 24 hours at 4°C, thus facilitating transport of samples (712).

Cell Culture Techniques

Probably, the most widely used method for primary isolation of MDV is inoculation of susceptible tissue cultures with blood lymphocytes or single-cell suspensions from lymphoid tissues of infected chickens. CKC and DEF cultures are preferred substrates for primary isolation of serotype 1 MDV; whereas CEF normally are used for isolation of viruses of serotypes 2 and 3 as well as for attenuated serotype 1 vaccine strains. Although CEF are less permissive for growth of low passage serotype 1 virus (152, 569, 715), some contemporary isolates appear to grow well in CEF, even on primary isolation (697). Cultures are inoculated with 1–2 x 10^6 cells, although some inhibition of viral plaque formation may be encountered with doses greater than 8 x 10^6 cells for some viruses (129). After 24–48 hours, the inoculum is washed off, and the culture is maintained under liquid or agar medium, usually without subculture.

Development of typical plaques (Fig. 15.3) in inoculated cultures within 3–12 days and the absence of such changes in comparable uninoculated (or sham inoculated) control cultures are evidence for isolation of MDV. The plaques induced by serotype 1, 2, and 3 viruses can be distinguished, with practice, by morphologic criteria (564, 689), but immunofluorescent staining with serotype-specific monoclonal antibodies provides a more accurate differentiation. Optimal time for observation of plaques varies with the cell substrate and serotype of the virus. MDV also has been isolated by direct culture of kidney cells from infected chickens or by inoculation of normal kidney cultures with trypsinized kidney cells from infected chickens (728).

Isolate Identification

MDV serotype 1 isolates should be free of contaminating MD vaccine strains. It normally is useful for the isolate to be plaque purified or cloned at the earliest possible passage. Serotype identity and purity can be confirmed using staining techniques with serotype-specific monoclonal antibodies (370). Pathotyping of serotype 1 MDVs, although not routine, may be accomplished by comparison of pathogenicity with that of prototype strains by inoculation of nonvaccinated chickens as well as chickens vaccinated with HVT or bivalent vaccines (699, 708). Freedom from extraneous viruses is also critical, because contamination with passenger viruses may alter the apparent pathogenicity of the isolate (316, 418, 677). Propagation of MDV isolates for up to 6 passages in CEFs or CKC cultures appears to exclude contaminants such as CIAV (738) and permits preparation of seed and working stocks, which can be more easily standardized and titrated. Although the possibility of attenuation during cell culture propagation must be considered, effects on pathogenicity are difficult to detect until after 20 cell-culture passages. To preserve virulence, some workers have preferred to propagate serotype 1 viruses *in vivo*, preparing stocks of cryopreserved spleen or buffy coat cells from infected chickens.

Virus Assay and Titration

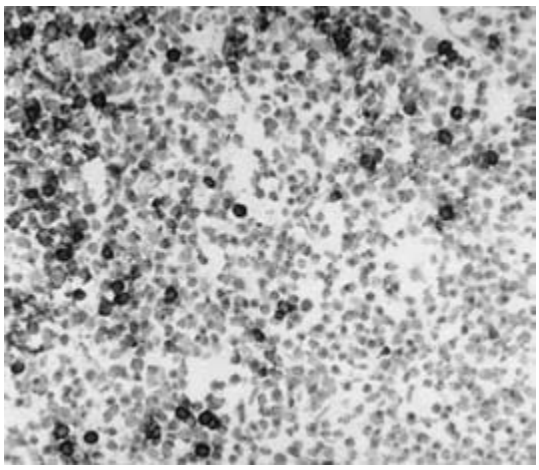
Viruses of serotypes 1, 2, and 3 can be assayed by *in vitro* techniques similar to those described for virus isolation. Methods differ for different serotypes, but most rely on plaque induction in susceptible cell cultures. Enumeration should be done as soon as plaques become mature (time varies with isolate), because secondary plaques may occur when cultures are maintained with liquid medium. Agar overlay methods have been described (635) but are not commonly used, probably because plaque formation is often delayed under agar and because secondary plaque formation under liquid medium has not been considered a significant problem. Procedures for titration of vaccine viruses have been reviewed (656) and are not fundamentally different from those for pathogenic isolates.

Viral Markers in Tissues

It is often desirable to detect the presence of viral infection in chickens without isolating the virus in culture. Such infection markers also have value for the identification of putative MDV isolates in cell cultures.

Viral Antigen Detection

MAb prepared against type-common and type-specific epitopes of all 3 MDV serotypes (370) are now used in preference to polyclonal antibodies for the detection of antigens in tissues. Viral antigens can be detected in feather tips and FFE, cytolytically infected lymphoid tissues, brain, or infected cell cultures with appropriate antibodies by fluorescent antibody tests (636), immunohistochemistry (134, 227), agar gel precipitin tests (242,



15.20. Marek's disease lymphoma showing expression of pp38 antigen (black) in some tumor cells. Immunoperoxidase staining with monoclonal antibody H19 and counterstaining with hematoxylin. ×450. (700) (Witter)

379), and enzyme-linked immunosorbent assays (178, 587). Antigen-positive cells are relatively rare in lymphomas and latently infected tissues, although pp38 and Meq have been identified frequently in the brain (228). In MD lymphomas, pp38-positive cells occasionally are observed (437) (Fig. 15.20), and Meq expression may be relatively common (557).

Polymerase Chain Reaction (PCR) Assays

The availability of the nucleotide sequences of different genes from a large number of viruses including the complete genome sequence of the 3 serotypes of MDV allows the use of PCR-based methods of specific detection of MDV. Primers designed to amplify 132 bp sequences specific to MDVs of serotype 1 have been described (49, 616, 750). PCR assays using these primers can discriminate between attenuated and wild-type strains and may detect viral DNA in lymphomas (177, 617). A wide variety of other primer sequences have also been used. However, PCR may not always be sensitive enough to detect latent infection due to the lower frequency of positive cells and the lower number of viral genomes per cell. qPCR assays using various primer sequences recently have been used to assay viral load in tissues from infected chickens (34, 87, 90, 302, 538) and are becoming essential tools for diagnosis and epidemiological studies of MD. For example, qPCR assays have been adapted for the detection of related MD vaccine strains (87) and PCR assays have been used to differentially detect MDV and HVT in both blood lymphocytes and feather tips of field chickens (248). Because clinical samples may contain inhibitors that can reduce DNA polymerase activity and produce false-negative results, an internal control is useful to establish the absence of such inhibitors. More recently, qPCR assays were carried out to study different aspects of MD biology including replication kinetics of MDV in feathers and lymphoid tissues (38, 303), correlation between protection and vaccine load (39), the association between replication rates of viral genomes and virulence (740) and the impact of specific gene deletions on replication (78). qPCR tests for the absolute quantitation of serotype 2 and serotype 3 MDV have also been developed (301, 543).

DNA Probes

Methods using DNA-DNA dot-blot hybridization with DNA probes for the detection of MDV DNA in feather tip extracts have been described (180). Furthermore, localization of virus-infected cells has been accomplished by *in situ* hybridization for both MDV (205, 553) and HVT (273).

Electron Microscopy

Herpesvirus particles can be detected by electron microscopy in the FFE and in productively infected cells *in vitro* (110).

Antibody Detection

Tests for identifying the presence of specific antibodies in chicken sera are useful in studies of viral pathogenesis and for monitoring SPF flocks. A number of procedures including agar gel precipitin, fluorescent antibody, enzyme-linked immunosorbent assays (747), and virus-neutralization tests are in common use; methods have been described (601). Commercial reagents are available only for the agar gel precipitin test, which is the least sensitive test but is adequate to detect serologic responses in infected or vaccinated chicken flocks. None of these tests, however, can differentiate antibodies of the 3 serotypes. The biologic significance of antibodies detected by different methods may vary (99).

Diagnosis of the Disease

Despite long-established guidelines for the pathologic diagnosis of MD (615), diagnosis of the clinical disease remains difficult in practice for several reasons. First, no truly pathognomonic gross lesion exists for MD. Gross lesions of MD can resemble those of other neoplasms and unrelated conditions characterized by tumors in visceral organs or grossly enlarged nerves. Finally, MDVs, ALVs, and REVs are widespread in commercial poultry, often resulting in simultaneous infections (176), complicating diagnostic efforts that depend on virological methods. The absence of a generally accepted confirmatory test makes even properly performed pathologic diagnoses subject to controversy. No currently accepted diagnostic standard exists for MD. However, the following model is suggested.

Diagnosis of MD must primarily be addressed by consideration of the characteristics of the proliferating cell populations that constitute the disease. Other disease-specific criteria, such as epidemiological factors, are also valuable. Virological criteria are less valuable but can help establish the presence of the causative agent or, more importantly, the absence of certain other agents. The process commences with the acquisition of a flock history and a sufficient number (5–10) of representative sick and dead chickens showing the lesions of the disease and proceeds as a series of steps.

Step 1—Clinical Data and Gross Pathology

Although enlarged peripheral nerves and visceral lymphomas are common in MD and one or both are invariably present, neither lesion occurs consistently nor is pathognomonic. Thus, other crite-

ria, such as age and lesion distribution, must be considered in the postmortem diagnosis of MD. Chickens may be diagnosed provisionally as MD if at least one of the following conditions is met: 1) leukotic enlargement of peripheral nerves; 2) lymphoid tumors in various tissues (liver, heart, gonad, skin, muscle, and proventriculus) in birds under 16 weeks of age; 3) visceral lymphoid tumors in birds 16 weeks or older that lack neoplastic involvement of the bursa of Fabricius; or 4) iris discoloration and pupil irregularity, as in Figure 15.9C. Proper examination of the bursa is particularly important and requires incision of the organ with close inspection of the epithelial surface. However, diagnoses based only on gross pathologic criteria are not definitive and additional steps are required.

Step 2—Histology, Cytology, and Histochemistry of Tumor Cells

Affected tissues, fixed in formalin or fresh-frozen, are used to prepare paraffin and cryostat sections, respectively. Impression smears of tumors may also be used. Essential diagnostic features may be seen in routine histologic sections stained with hematoxylin and eosin or touch preparations stained with methyl green pyronin or Shorr's stain (615). Cellular and viral antigens can be demonstrated in frozen or, in some cases, paraffin sections by immunohistochemistry. A mixed population of small to large lymphocytes, lymphoblasts, plasma cells, and macrophages are typically found in MD tumors and nerve lesions (484). The proportion of cell types varies with the stage of the disease and virulence of the virus, and the most aggressive lymphomas may contain high numbers of lymphoblasts. MD tumors are a mixed population, and most cells express MHC class II antigen and T-cell markers, especially CD4 (although $CD8^+$ cells may also be present) (577), but IgM is present on less than 5% of cells. CD30 is also commonly found in MD tumor cells (557). MATSA (731) can be demonstrated with polyclonal or monoclonal (369) antibodies on 5–40% of MD tumor cells but is also present on activated T cells (412). Tumor lesions tentatively identified as MD in step 1, which are composed of pleomorphic lymphoid cells that predominately express CD4/CD8 markers and where expression of IgM or B-cell markers is limited or absent, may be diagnosed provisionally as MD. Demonstration of CD30 or MATSA may potentially be useful, but more data are needed.

Step 3—Virologic Criteria

For tumors that satisfy MD criteria listed in steps 1 and 2 or for atypical tumors, the association of MDV with the tumor cell is a useful confirmatory procedure. Viral antigens such as pp38 or Meq may be detected in tumor cells by immunohistochemistry or fluorescent antibody tests (437, 557), but because neither antigen is expressed in all cells or in all tumors, negative results are less helpful than positive results. Also, very small numbers of positive cells may reflect the presence of inflammatory T cells, incidentally infected with MDV, which are unassociated with the primary tumor. *In situ* hybridization is another technique to localize MDV genome in tumor cells (557). PCR assays may also detect MDV DNA in tumors, especially in tissues with high concentrations of tumor cells (e.g., tumor nodules). PCR assays on other tissues and virus isolation from buffy coat or spleen cells demonstrate the virus in the bird but do little to associate the virus with the tumor cells. Similar information is provided by antibody tests. There may be a quantitative association between virus load and MD tumors. Low levels of virus or viral DNA may be detected in lymphocytes from nontumor-bearing chickens, but most tumor-bearing chickens have high viremia titers (729) and are usually PCR positive (177, 624). Therefore, the possibility that infection criteria in excess of some arbitrary threshold level, as yet undetermined, would better correlate with lymphomatous lesions could be explored. Thus, the demonstration of high quantities of virus or viral antigens in tumor cells at the exclusion of other relevant tumor viruses should be sufficient, along with other criteria in steps 1 and 2, to establish a diagnosis of MD.

Applications

Steps 2 and 3 do not invariably need to be performed in sequence. Because PCR assays are quick to perform, establishing whether single or multiple tumor virus infections are present can, in some cases, help determine additional procedures to be conducted. However, characteristics of the tumor itself should be paramount.

Pathotyping of MDV Strains

The concept of MDV pathotypes has arisen from the recognition of the existence of strains that are associated with increased virulence, which show correlation with breaking of vaccinal immunity in the field (708). Methods to differentiate between classical strains of MDV and the more virulent pathotypes have been examined. However, the ADOL (Avian Disease & Oncology Laboratory) method of pathotyping, based on the induction of lymphoproliferative lesions in chickens vaccinated with different vaccination regimes, has been the most widely used. This method was used to characterize more than 45 isolates into distinct vMDV, vvMDV, or vv+MDV pathotype groups (699). Even though ADOL method stipulates the use of line 15×7 chickens for pathotyping, experiments with other lines of birds also have given similar results (98, 708).

Differential Diagnosis

Lymphoid leukosis is a clonal, bursal lymphoma induced by ALV and, under some conditions, by REV in chickens older than 16 weeks of age. Chickens usually have gross tumors in the bursa of Fabricius, and tumor cells are uniform, blast-like, and pyroninophilic and express B-cell markers and IgM. Also, the tumor cells have clonal insertions of proviral DNA near the *c-myc* gene (see "Leukosis/Sarcoma Group"). Nerve enlargements, runting, and nonbursal T-cell lymphomas can be induced by REV but, thus far, have only been observed under experimental conditions or where chickens have been inoculated with contaminated vaccines. Lymphocytes obtained from REV-induced nerve lesions or tumors are not known to express pp38 or Meq. Cells from nonbursal RE lymphomas are negative for MHC class II and predominantly stain for CD8 antigen (162). (See "Reticuloendotheliosis.") Exclusion of ALVs or REVs, where possible, through negative PCR, histochemical assays on tumors, or antibody tests may provide strong support for a diagnosis of MD when other MD-related criteria are positive.

Peripheral neuropathy is a neurological disease of uncertain etiology that causes paralysis and nerve enlargement in a low proportion of commercial chickens 6–12 weeks of age (31, 321) and has been described in commercial (704) and SPF chicken flocks (67). Affected chickens lack visceral lymphomas; the nerve lesions are uniformly B-type; and MDV is rarely, if ever, demonstrated. Other diseases that may present confusing gross lesions or paralytic signs are myelocytomatosis (myeloid leukosis), myeloblastosis, erythroblastosis, carcinoma of the ovary, various other nonviral neoplasms, riboflavin deficiency, tuberculosis, histomoniasis, genetic gray eye, Newcastle disease, avian encephalomyelitis, and joint infections or injuries. Myeloid leukosis is a common tumor in broiler breeder flocks that superficially resembles MD but can be differentiated histologically. The tumor cells are myeloid in nature and lack T cell and MD viral markers. Multicentric histiocytosis (240) is a disease of broiler chickens characterized by enlarged mottled spleens and lesions in other viscera composed mainly of histiocytes. No lymphocytes were identified, and MDV was not demonstrated in tumors, although an association with subgroup J ALV has been suggested (24).

Diagnosis of Other MD Syndromes

Transient paralysis occasionally is observed in the field, especially in chickens not vaccinated against MD. Most cases represent the classical form in which chickens experience flaccid paralysis of neck or limbs for 1–43 days and then undergo a complete recovery. This syndrome can be differentiated from the neurological form of MD (fowl paralysis) by its transient nature and flaccid rather than spastic paralysis. Transient paralysis can be differentiated from peripheral neuropathy by its transient nature and the absence of enlarged peripheral nerves with B-type lesions. Davidson *et al.* (180) differentiated transient paralysis from peripheral neuropathy on the basis of PCR tests for MDV on brain tissue; however, brains from MDV-infected chickens without transient paralysis may also be detected as positive by PCR assays (710). In contrast, detection of viral antigens in the brain appeared to correlate with the onset of paralytic signs (228).

Skin leukosis (the skin form of MD) can be differentiated from dermal squamous cell carcinoma, which is commonly observed in defeathered broiler chickens at processing (241). MD lesions are nodular and contain lymphoid cells, whereas squamous cell carcinomas have a craterlike gross appearance and are composed of squamous epithelial cells. Both occur in feather tracts in association with feather follicles (361).

Lymphodegenerative lesions of lymphoid organs characterized by organ atrophy and immunosuppression are difficult to diagnose, because many diseases produce similar changes. However, lymphoid organs atrophied consequent to productive infection with MDV may also contain MD viral antigens, such as pp38, at least for a short period.

Intervention Strategies

The development of successful vaccines for control of MD (157, 459, 546) was a significant achievement. Vaccination represents, for now and the foreseeable future, the central strategy for the prevention and control of MD. Genetic resistance and biosecurity, however, are critical adjuncts to vaccination. No effective practical treatment exists for the disease in individual chickens or infected flocks. An integrated strategy to prevent early infection, to slow the acquisition of virulence of field strains, and to provide superior immune responses seems most likely to succeed (701). Detailed reviews on MD vaccines and control procedures are available (82, 566, 703).

Vaccination

Types of Vaccines

Several different types of MD vaccines are in common use, both individually and in various combinations. The most widely used products are low pathogenic serotype 1 MDV (545, 546), naturally avirulent HVT (459), and serotype 2 viruses (571, 725). The serotype 2 strains usually are combined with HVT to take advantage of the synergistic activity documented between serotypes 2 and 3 (574, 688). All vaccine types are protective but to varying degrees. HVT, mainly strain FC126 (718), continues to be extensively used because it is effective and economical to produce and combines well with other products. Although both cell-free and cell-associated forms of HVT are available, the latter has been most widely used because it is more effective than cell-free virus in the presence of maternal antibodies (707). Bivalent vaccines based on combinations of HVT and either SB-1 (571) or 301B/1 (692) strains of serotype 2 MDV were introduced in the mid 1980s. The CVI988 strain (546) used in The Netherlands (403) and other countries since the early 1970s, was introduced to the United States in the early 1990s. Another attenuated serotype 1 strain, R2/23 (694), was also introduced in the 1990s. Serotype 1 and 2 vaccines are available only as cell-associated products.

Vaccine Administration

MD vaccines are administered to chicks at or before hatch because early immunity is essential. Both cell-associated and cell-free vaccines are given by subcutaneous or intramuscular inoculation at a dose usually in excess of 2000 plaque-forming units per chick. Vaccines are also effective when administered to embryos at ED 18 (603). *In ovo* vaccination is now performed by automated technology (317) and is widely used for vaccination of commercial broiler chickens, mainly because of reduced labor costs and greater precision of vaccine administration. Deposition of the vaccine by the amniotic or intraembryonic route is essential for optimal protection (680). Proper handling of vaccine during thawing and reconstitution is crucial to ensure that adequate doses are administered (245, 306).

Factors Affecting Efficacy

Vaccines typically are given at doses of 2000–6000 plaque forming units per chick, but these may be significantly reduced in broilers. Higher doses (196, 689) or revaccination (40) offered little improvement. Revaccination at 7–12 days continues to be popular in Europe and occasionally is used in the United States, but the effectiveness of this procedure has not been validated by laboratory studies. Maternal antibodies reduce the ef-

fectiveness of cell-associated vaccines but do not abrogate the protective effect (131). Vaccination of breeders with a serotype 1 or 2 virus leaves progeny more responsive to vaccination with HVT (346).

The shorter the interval is between vaccination and exposure to the virulent field virus, the poorer the level of protection (459). Early exposure is undoubtedly one of the most important causes of excessive MD in vaccinated flocks because field exposure usually occurs very soon after placement of chickens (717) and because at least 7 days is required to establish solid immunity after vaccination (45). The vaccine strain of virus also has a major influence on vaccine efficacy. Immunity induced by weaker vaccines such as HVT may be excellent against low virulence challenge but can be completely overwhelmed by early challenge with highly virulent strains (699). Although high virulence strains commonly are invoked to explain field outbreaks of disease, many alternate causes should be considered.

The strain of chicken is also an important determinant of vaccine efficacy. Schat *et al.* (574) found that HVT vaccine in genetically resistant chickens resulted in a stronger immunity than did the bivalent (HVT + SB–1) vaccine in susceptible chickens.

Stress appears to interfere with the maintenance of vaccinal immunity. In chickens properly vaccinated at hatch and well protected following challenge, Powell and Davison (507) induced MD lesions and mortality by immunosuppressive treatment at 10 weeks of age. The possibility that immunosuppressive stress may play an important role in MD outbreaks in vaccinated flocks, especially those that occur after the onset of egg production, deserves consideration (703). IBDV (597), REV (713), reoviruses (547), and CIAV (469, 470, 739) have been reported to interfere with the induction of vaccinal immunity, although very specific conditions are sometimes required.

Vaccination Strategies

Efficacy data that compare certain groups of vaccines are available (citations in 566, 691, 703). Bivalent serotype 2+3 vaccines and R2/23 are clearly more effective than HVT (695). The most effective vaccine, however, appears to be the original CVI988 vaccine (695), a result consistent with earlier reports from Europe (669). Efficacy rankings of vaccines, however, have not always been reproducible when performed in different laboratories and should be interpreted with caution.

MD vaccines as a class are unusually effective, often achieving greater than 90% protection under commercial conditions (703). However, attention is often focused on flocks in which MD losses are perceived to be excessive (305, 357, 744). Causes for such vaccine failures are difficult to ascertain by retrospective analysis (703), although early exposure and the emergence of new MDV strains with increased virulence may be important.

The propensity of MDVs to evolve to greater virulence (699) is critical to the strategic use of vaccines for MD control. Vaccination itself no doubt contributes to this virulence increase, which, in turn, tends to make earlier vaccines obsolete. Kreager (359) has noted that the useful life of a MD vaccine has been about 10 years under current management conditions. Although this is perhaps an overstatement, the implications are serious. Since CVI988 was introduced in the United States, some evidence already suggests that contemporary strains have increased their virulence in CVI988-vaccinated chickens (702).

Choice of a vaccine program is an important consideration for MD control. A rational approach is to use the least effective product required to control the disease on a particular farm at a particular time (700). In practice, HVT alone provides adequate protection to many broiler flocks under normal conditions. Broiler flocks, especially during winter months, and layer/breeder flocks often require bivalent (serotype 2+3) vaccines rather than HVT alone. Where these vaccines are insufficient, the CVI988 strain is used. Mixtures of CVI988 with HVT or both serotype 2+3 vaccines are also used, although little evidence exists for synergistic activity of CVI988 with other vaccine serotypes (714).

Research continues to be directed toward the development of improved vaccines through recombinant DNA approaches (551), but with limited practical success. Recombinant fowl poxvirus (374, 445), HVT (552), and MDV (268) vaccines expressing various MDV genes have shown some protective efficacy. The rFPV vaccines were effective to some degree in chickens positive for maternal antibodies to MDV, but were not tested in chickens with maternal antibodies to FPV. Incorporation of cytokines in vaccines in recombinant vaccines may improve vaccine efficacy as suggested by the inclusion of the gene for avian myelomonocytic growth factor in FPV (189). However, the lack of protection with rFPV vaccines expressing avian influenza genes against avian influenza in birds with antibodies to FPV (642) suggests that rFPV vaccines are unlikely to yield improved MD vaccines.

Some progress has been made with deletion mutant vaccines. MDV strains lacking vIL-8 (167) or with mutations in the CtBP-interaction domain of the *meq* (78) were protective against challenge with vv or vv+ MDV. In a limited experiment, DNA vaccination using pBAC20, derived from attenuated MDV, provided limited protection against challenge (658).

Efforts to derive more efficacious serotype 1 vaccines through classical virological approaches (702) or retroviral insertion (716) yielded several promising candidates. However, these strains did not protect better than the best commercial CVI988 vaccines (711). These authors questioned if the efficacy of MD vaccines is limited by some type of biological threshold.

The emergence of increasingly virulent viral strains, coupled with an apparent reduction of vaccine efficacy during the past 20 years, has prompted justifiable concern. This suggests that vaccination by itself does not provide a complete control program and is not the ultimate solution for MD. Strict biosecurity procedures to reduce early exposure and the presence of genetic resistance are essential adjuncts to a successful vaccination program.

Genetic Resistance

The well-known variation in susceptibility of different lines of chickens to MD challenge is determined by genetic factors (159) and provides a unique opportunity to consider genetic approaches to the control of MD. Indeed, poultry breeders have included resistance to MD in selection programs for many years. However, genetic resistance can be overcome by challenge with

highly virulent MDVs and is best applied in concert with vaccination and biosecurity to achieve optimal control. Literature on genetic resistance against MD is extensive (see reviews 25, 86). Genetics influences virtually every aspect of host response to MD. However, only those issues germane to disease control programs are considered here.

Characteristics of genetic resistance to MD are generally favorable for inclusion in selection programs by primary breeders. The magnitude of the effect, at least under certain conditions, is large enough to have considerable economic impact (159). Resistance is not associated with undesirable production traits (68) and in one study (15) was correlated with higher egg production and egg weight. Variation of MD susceptibility in single-sire families indicated that sufficient heterogeneity exists to warrant selection for resistance in commercial chickens (68), which is still the case in recent commercial genetic stock (204). Moreover, estimates of the heritability of resistance, although variable, were often relatively large (reviewed in 25).

Selection Methods

Selection programs for resistance historically have been based on progeny testing or family selection (158) or reproduction from survivors of exposed breeding flocks through mass selection (402). Resistance traditionally has been measured by challenge of unvaccinated chickens with virulent MDV, but recent studies (26) conclude that resistance may better be determined in vaccinated stocks. Acquisition of resistance consequent to selection can occur rapidly. Maas *et al.* (402) reduced susceptibility from 76% to 8% in 6 generations upon mass selection. Probably the most dramatic example was the development of lines N and P from Cornell Randombred control stock by family selection, resulting in divergent susceptibilities of 4% and 96%, respectively, after 4 generations (158). Family selection may be more appropriate than mass selection for commercial breeders in order to avoid high loss of genetic material on initial challenge exposure (25).

Selection based on blood typing relies on the close relationship between MD resistance and certain alleles of the B-F region of the MHC, especially B^{21} (76, 77). In theory, such a selection procedure may simplify production of resistant stocks in populations containing a specific allele for resistance although the possibility of negative associations with productivity traits needs to be measured (232). Furthermore, the value of selection for MHC-associated markers may vary considerably among commercial lines and crosses (69, 252).

Evidence that non-MHC genes may also be involved in resistance is provided by the observation that RPL line 6 and line 7 chickens, which are both homozygous at the B locus for the B^2 allele, differ markedly in MD susceptibility (166). Furthermore, non-MHC effects were considered more important than MHC effects in studies on several commercial lines (236). Studies to identify and map quantitative trait loci associated with MD resistance may provide new tools to enhance selection programs for MD resistance. Up to 14 or more putative quantitative trait loci have been identified (85, 665, 737), and some of these presently are being evaluated in commercial stocks. Using microsatellites, McElroy *et al.* (413) identified several markers associated with resistance to MD. The combination of QTL analysis and microarrays (392) or the use of 2-hybrid screen approaches (448) may further facilitate the identification of useful markers for selection. Resistance has been considered dominant, although this varies to some extent (258), and, in most cases, resistance of crosses has been intermediate to that of the parent strains (69, 103).

Applications to Control

The knowledge that genetically resistant chickens are protected by vaccination to a greater extent than more susceptible strains (637) has fueled interest by commercial breeders to emphasize MD resistance in selection programs. However, synergy between host genetics and vaccines is complex. Some resistant B-haplotypes were demonstrated only by challenge of previously vaccinated chickens (26). However, the relative efficacy of MD vaccines is also influenced by B-haplotype (27). Serotype 1 vaccines provided the best protection for certain B-haplotypes, but serotype 2 vaccine was best for B^5 chickens (28), suggesting that one could select the most appropriate vaccine based on the predominant B-haplotypes in a particular strain (29). In practice, this issue has been either ignored or addressed through the use of vaccines containing multiple serotypes.

In light of the selection tools available, the absence of negative correlations, and the major benefits to be derived, it is not surprising that some breeders place a high priority on this approach. Although selection for B-haplotype has been practiced with variable success and has proven to be complex, especially in meat strains (416), breeders acknowledge the value of improved genetic resistance to offset virulence increase by viral strains and the limitations of current vaccines (357).

Management Procedures

Strict biosecurity practices to limit the extent of early MDV exposure, although impractical as a primary control procedure, are a crucial and cost-effective adjunct to vaccination. MD control is compromised because modern poultry management too often places replacement flocks of different ages in close proximity to each other or requires the reuse of litter from a previous broiler flock. The failure to prevent early exposure is perhaps the most important single cause of vaccine failures. Improved hygiene has often appeared to play a key and cost-effective role in the elimination of excessive MD losses in vaccinated flocks. However, management decisions in commercial poultry operations appear linked to cost analyses and procedures advantageous for disease control are not always implemented (356). Relevant sanitation principles have been reviewed (57, 478).

For SPF flocks, higher standards of biosecurity are required and become cost effective. Most SPF operations rely on the use of filtered-air, positive-pressure houses (20, 192), which, along with strict biosecurity measures, successfully can maintain large flocks free of MDV infection for long periods. In this case, biosecurity becomes a substitute for vaccination and provides a practical demonstration that MDV can be eradicated in certain flocks, at least under specialized conditions.

Nononcogenic Turkey and Chicken Herpesviruses

Turkey herpesviruses and serotype 2 MDV are not recognized as pathogens in avian hosts. Interest in these viruses derives mainly from their use to immunize chickens against MD. However, both viruses occur in nature independent of vaccination, and it seems appropriate to also consider some aspects of their epizootology and pathogenesis in their natural or alternate avian hosts that have not been addressed elsewhere in this chapter. Review papers by Calnek (104, 107) may be consulted for additional details on pathogenesis of these infections.

Turkey Herpesvirus (HVT)

HVT was isolated from normal turkeys by Kawamura *et al.* (338) and Witter *et al.* (718). The virus is endemic and ubiquitous in domestic turkeys (726). Isolations have also been reported from wild turkeys (161). In chickens, the virus has also become ubiquitous because of its widespread administration to day-old chickens to prevent MD. The genome of HVT showed close structural and sequence similarity (11, 347) to that of serotype 1 strains even though distinct genes do exist in each of these viruses. Analysis of the functions of HVT genes will be facilitated with the recent success in the construction of BAC clones of HVT (35).

In turkeys, the virus spreads rapidly through exposed flocks, presumably by contact exposure. Virtually all individual turkeys become viremic and develop antibodies within a few weeks (726). The virus appears to mature in the FFE, because cell-free skin extracts are infectious (727) although viral antigen was found only infrequently and at low levels in the FFE of infected turkeys (212). No vertical transmission has been demonstrated (726). HVT may be transmitted from turkeys to chickens under experimental conditions (727), which is probably rare in the field. Only limited contact spread occurs among chickens (144, 146) but transmission could not be demonstrated by the airborne route (140). Replication of virus in the FFE of infected chickens appears limited and transient (111, 139, 751). The virus also appears to replicate less efficiently than MDV in skin (519), although increased levels of HVT DNA were observed in FFE of HVT-vaccinated chickens after MDV challenge (386).

Fabricant *et al.* (212) studied the early pathogenesis of HVT infection in chickens and turkeys. Chickens had no cytolytic infections in any lymphoid organ. Turkeys infected with HVT did have some viral antigen-positive cells at 4–14 days post exposure in the spleen, but no cytolytic infections in bursa or thymus were seen. In chickens, there was no depression of bursa or thymus size, although a transient splenomegaly was variably present (111, 212). B cells are rarely infected, but latent infection is probably established in MHC class II-positive T cells (104). Holland *et al.* (274, 275) detected expression of gB in the spleen, to a lesser degree in the thymus, and to very limited degrees in the bursa of infected chickens; nervous tissue was uniformly negative. NK cell activity was stimulated through at least 8 week PI (605). HVT can be recovered from infected chickens for long periods, and antibodies persist for life (525, 723). The virus is apparently nononcogenic in turkeys (687, 718), but the possibility of fertility problems in HVT-infected toms has been raised (10, 657). The virus generally causes no clinical disease in intact or immunosuppressed chickens (606, 718) and is not normally detrimental to the immune response (220). However, atrophy of the bursa and thymus was noted following administration of high doses (244), and minor cellular infiltrations in nerves were observed (212, 723). In contrast, when S-line chickens were infected with HVT *in ovo* (ED 8) and then hatched and raised, up to 19% developed clinical paralysis and gross nerve enlargement due to inflammatory type lesions were observed (121). Chickens exposed to HVT at ED 14 or earlier showed higher incidence of immunological tolerance resulting in a persistent HVT viremia (748). A possible role for HVT as a predisposing factor in autoimmune disease is suggested by its implied involvement in peripheral neuropathy (31) and autoimmune vitiligo (207); both syndromes are restricted to certain strains of chickens.

Serotype 2 Marek's Disease Virus

Apathogenic strains isolated from clinically normal chickens (63, 143) subsequently were determined to be a separate serotype on the basis of FA and AGP tests (675, 676). Natural infection with such strains provided protection against MD challenge (63, 742). Other unique features of this virus group were further elucidated following the isolation of the SB-1 strain (571). Prior to their widespread use as vaccines, serotype 2 viruses were common, although not universal in commercial chicken flocks in England and the United States (63, 690). Avirulent isolates were also prevalent in Australia (517). However, the epidemiology has been complicated by the artificial distribution of the virus in the United States through a seeder chick program (742, 743) and through nearly 20 years of administration of serotype 2 vaccines (123, 722). The virus must now be considered ubiquitous in chickens. Chickens appear to be the only natural host, although apathogenic isolates that resembled the HN strain were isolated from Japanese silkies, red jungle fowl, and Ceylon jungle fowl reared in a zoo (145).

Serotype 2 viruses spread readily by contact (571, 725) and replicate in the FFE (142). Following inoculation of day-old chickens, the virus can be first isolated 5–6 days PI (111). The virus reaches peak titers at 2–4 weeks and persists for long periods (111, 725). Antibodies are induced readily and persist.

A transient splenomegaly was induced between 4–12 days after inoculation of chicks with the SB-1 strain, but no bursal atrophy and only occasional thymic atrophy was seen and cytolytic infection of lymphoid organs was not observed (111). In contrast, Lin *et al.* (391) found that viral antigens were expressed in spleen and bursal tissues 5–14 days after infection, primarily in B cells, but no gross or microscopic changes were observed. Calnek (104) considered B cells and macrophages relatively refractory to infection, and the cells supporting latent infection lacked MHC class II antigens, thus differing from those in HVT infections. However, T cells may not be very susceptible either because $CD4^+$ and $CD8^+$ cells from SB-1-infected chickens induced few, if any, plaques in cell culture assays (376). Certain subpopulations of bursal cells appear to support latent infections that interact with ALV, and serotype 2 was isolated from a B lymphoblas-

toid cell line (262). SB-1 did not suppress humoral immunity (200) and is not normally considered immunosuppressive. However, Friedman *et al.* (220) found a diminished response to a B lymphocyte-specific mitogen and decreased antibody responses to bovine serum albumen in chickens vaccinated with the SB-1 strain in combination with HVT.

The SB-1 strain did not produce neoplastic lesions in either normal or immunosuppressed chickens, but because some cytolytic lesions were noted in immunosuppressed chickens, the virus was designated nononcogenic rather than apathogenic (571). Also, a variety of lesions were induced by *in ovo* inoculation of SB-1, but none were neoplastic (121, 571, 715). The absence of lymphoma induction has been confirmed by other workers (63, 141, 725) but Pol *et al.* (506) described visceral lymphomas in 2 of 48 chickens inoculated with the HPRS-24 strain.

Vaccination with serotype 2 viruses causes a pronounced enhancement of B cell lymphomas in certain genetic strains of chickens exposed at an early age to subgroup A ALV (30) or REV (14). Salter *et al.* reported that spontaneous lymphomas are also enhanced by SB-1 exposure (see "Leukosis/Sarcoma Group"). Apparently, the subpopulation of B cells susceptible to transformation is also uniquely susceptible to infection by serotype 2 MDVs but not by HVT (222). The ability of serotype 2 virus to enhance LL was attenuated without abrogation of its protective properties against MD challenge (696). ALV (subgroup A) has now been eradicated from most of the chicken lines susceptible to serotype 2 enhancement and field problems due to serotype 2 enhancement of LL are rare (see "Leukosis/Sarcoma Group").

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Leukosis/Sarcoma Group

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Introduction

Definition and Synonyms

The leukosis/sarcoma (L/S) group of diseases designates a variety of transmissible benign and malignant neoplasms of chickens caused by members that belong to the family Retroviridae (210). Lymphoid leukosis has been the most common form of L/S group of diseases seen in field flocks, although myeloid leukosis has also become prevalent. The neoplasms and their synonyms are listed in Table 15.5. Members of this group of avian viruses are characterized, as are all members of the Retroviridae, by possession of an enzyme reverse transcriptase, which directs the synthesis of the proviral DNA form of the RNA virus that forms part of the retroviral life cycle and from which the family name is derived. These avian retroviruses, including avian leukosis virus (ALV)-related viruses, formerly were placed in a subgenus termed avian type C oncornaviruses (329), but they recently have been termed alpharetroviruses (210). Members of this group of viruses have similar physical and molecular characteristics and share a common group-specific antigen.

Because of the relationships between these viruses (145), they are discussed as a group in most parts of this chapter. Sections reflecting the host response ("Pathology" and "Pathogenesis") are discussed under the pathologic entities without regard for the properties of the inducing virus (es) other than their inclusion in the L/S group.

Economic Significance

Infection of chickens with ALV is the most common L/S virus infection encountered in field flocks and is known to be of significant economic importance. Economic losses from ALV-induced diseases are attributed to 2 sources. First, tumor mortality commonly amounts to around 1–2% of birds, with occasional losses of up to 20% or more. Second, subclinical infection by ALV, to which most flocks are subject, produces a depressive effect on a number of important performance traits, including egg production and quality (226, 228). Economic losses due to ALV tumor mortality and reduced productivity are estimated to be in millions of U.S. dollars each year. In 1991, the benefits to a major

Table 15.5. Neoplasms caused by viruses of the leukosis/sarcoma group.

Neoplasm	Synonyms
Leukoses	
Lymphoid leukosis	Big liver disease, lymphatic leukosis, visceral lymphoma, lymphocytoma, lymphomatosis, visceral lymphomatosis, lymphoid leukosis
Erythroblastosis	Leukemia, intravascular lymphoid leukosis, erythroleukosis, erythromyelosis, erythroblastosis, erythroid leukosis
Myeloblastosis	Leukemic myeloid leukosis, leukomyelosis, myelomatosis, myeloblastosis, granuloblastosis, myeloid leukosis
Myelocytoma(tosis)	Myelocytoma, aleukemic myeloid leukosis, leukochloroma, myelomatosis
Connective tissue tumors	
Fibroma and fibrosarcoma	
Myxoma and myxosarcoma	
Histiocytic sarcoma	
Chondroma	
Osteoma and osteogenic sarcoma	
Epithelial tumors	
Nephroblastoma	Embryonal nephroma, renal adenocarcinoma, adenosarcoma, nephroblastoma, cystadenoma
Nephroma	Papillary cystadenoma, carcinoma of the kidney
Hepatocarcinoma	
Adenocarcinoma of the pancreas	
Thecoma	
Granulosa cell carcinoma	
Seminoma	Adenocarcinoma of the testis
Squamous cell carcinoma	
Endothelial tumors	
Hemangioma	Hemangiomatosis, endothelioma, hemangioblastomas, hemangioendotheliomas
Angiosarcoma	
Endothelioma	
Mesothelioma	
Related tumors	
Osteopetrosis	Marble bone, thick leg disease, sporadic diffuse osteoporostitis, osteopetrosis gallinarum
Meningioma	
Glioma	

egg-laying breeder company from successful eradication of ALV infection were estimated to be $15 million per year (92). During the 1990s, the broiler breeder industry identified an ALV-induced myeloid leukosis (377) as its highest disease priority, as losses due to this disease (485, 491) have threatened the economic viability of the entire broiler industry (529).

Public Health Significance

Recent studies have addressed the relationship between avian tumor viruses, particularly ALVs, and human health. Evidence for the presence of antibodies to ALVs in humans usually has been lacking or at best is presumptive (276). However, using enzyme immunoassays and Western blots, low-titer antibodies to ALVs were detected in sera from poultry workers (277–279). Endogenous and exogenous ALV also were detected in the albumen of commercial eggs using reverse transcription polymerase chain reaction (397, 398), but the public health significance was not determined. In a serological survey that included 549 human subjects, including groups exposed and not exposed to chickens, significant differences between men and women were found for the prevalence of antibodies to ALV but were not related to exposure to chickens (100). Robertson *et al.* (423) discussed the significance of detecting reverse transcriptase activity in vaccines derived from chicken cells. Reverse transcriptase activity was detected in all chicken cell-derived measles and mumps vaccines, suggesting the presence of endogenous ALV elements; however, no evidence of antibodies or proviral sequences of ALV was found in the vaccines or the sera from vaccine recipients (266, 268, 457, 519). Clearly, no strong unequivocal evidence suggests that ALV constitutes a danger to public health.

History

The earliest reports of leukotic diseases in fowl are those of Roloff (428), who described a case of "lymphosarcomatosis" in 1868, and of Caparini (88), who in 1896 described cases of "fowl leukemia." In 1905, Butterfield (81) diagnosed "aleukemic lymphadenosis" in the United States. In 1908, Ellermann and Bang (177), working in Copenhagen, founded the discipline of viral oncology when they transmitted erythroleukemia and myeloge-

nous leukemia by inoculation of chickens with cell-free filtrates. The significance of their discovery was not fully appreciated, because at that time leukemia was not recognized as a neoplastic disorder. Ellermann and Bang also proposed the word "leucosis" to designate leucemic and aleucemic cases.

Ellermann also provided further classification of the pathological forms of avian leukosis that essentially still holds today (176). He described in his monograph "The Leucosis of Fowls and Leucemia Problems," (176) (1) "lymphatic leucosis," with lymphoblastic hyperplasia; (2) "myeloic leucosis," with leukemia and general hyperplasia of "myeloic" cells (myelocytes, "large mononuclear cells," and "poikilonuclear cells"); and (3) "intravascular lymphoid leucosis," involving "lymphoidocytes," which he concluded were erythrocytic cells and that this intravascular form was erythroleukosis.

Also in the early decades of the twentieth century, Rous, working in New York, was undertaking studies on the transmissibility of avian sarcomas. In 1909, he suceeded in transplanting a spindle cell sarcoma from one hen to another (432) and soon after showed that the transplantable tumor could be transmitted by cell-free filtrates (431). Over the next two decades, some 20 transplantable tumors of fowl were shown by a number of workers to be filterable (103). As with the leukoses, however, doubt existed about the nature of these avian tumors and their relevance to malignant neoplasms in mammals.

In the 1920s and 1930s, many transmission studies were also conducted on the avian leukoses by notable workers including Furth (222) in the United States, Jérmai (275) in Hungary, Engelbreth-Holm (179, 180) in Denmark, and Oberling and Guérin (358) in France, and numerous strains of avian leukosis virus were isolated (75). An important question was whether the same or different agents caused the 3 forms of leukosis. In general, erythroid and myeloid leukosis were readily transmissible, either in pure or mixed forms, lymphoid leukosis was not. Furth provided evidence of transmission of lymphoid leukosis with filtrates (222), and conclusive proof of the viral etiology of this form came from transmission experiments of Burmester and his coworkers in 1946–1947 (66, 68, 69).

Much of the early research on avian leukosis and sarcomas was motivated by basic scientific and medical interest. From 1920–1940, however, the expansion of the poultry industry in the United States and elsewhere brought increasing losses from the so-called "avian leukosis complex." Research was carried out particularly at land-grant colleges and state agricultural stations in the United States, with the aim being practical control of these diseases (75). The disease picture was complicated by the inclusion in the leukosis complex of neurolymphomatosis (range or fowl paralysis), and visceral lymphomas associated with this disease, which were increasing in prevalence. There was much uncertainty and argument as to whether or not neurolymphomatosis (now termed Marek's disease) was caused by the agents that caused the leukoses (376). The use of the term "visceral lymphomatosis" (282) to cover both lymphoid leukosis and lymphomas associated with neurolymphomatosis did not help the debate (44, 85). In 1939, the U.S. Department of Agriculture established the Regional Poultry Research Laboratory at East Lansing, Michigan, (later renamed the Avian Disease and Oncology Laboratory) to study the cause and control of fowl paralysis (and other neoplastic conditions). In 1959, a similar center, the Leukosis Experimental Unit of the Houghton Poultry Research Station, was established in England. It is known now, from work at these and other centers, that the 3 forms of leukosis, caused by retroviruses, are distinct from the neural and visceral forms of Marek's disease, caused by a herpesvirus.

Numerous other solid tumors have been associated with the avian leukoses occurring in the field and in transmission experiments, including connective tissue tumors, nephromas and nephroblastomas, various other epithelial tumors, endothelial tumors, and neural tumors (34, 204). Also included in the avian leukosis complex is a hypertrophic bone disorder osteopetrosis, first reported by Pugh in 1927 (407) and described and reproduced in 1938 by Jungherr and Landauer (283). The latter workers, and later Burmester and coworkers (66, 215), noted the association of osteopetrosis with lymphoid leukosis.

A rapid increase in knowledge of the leukosis/sarcoma group and the causative viruses occurred after 1960, when tissue culture techniques were developed for studying avian tumor virus-host interactions at the cellular level. The use of Rous sarcoma virus (RSV) as a model to study neoplastic transformation was largely responsible for this. A number of important biological phenomena were discovered by biomedical research groups, most notably those of H. Hanafusa, H. Rubin, H. M. Temin, and P. K. Vogt, which led to knowledge of the biochemistry and molecular biology of avian retroviruses and which benefited the work of the avian disease research laboratories.

More detailed reviews of the history of avian retrovirus research are available elsewhere (75, 164, 374, 534).

Etiology

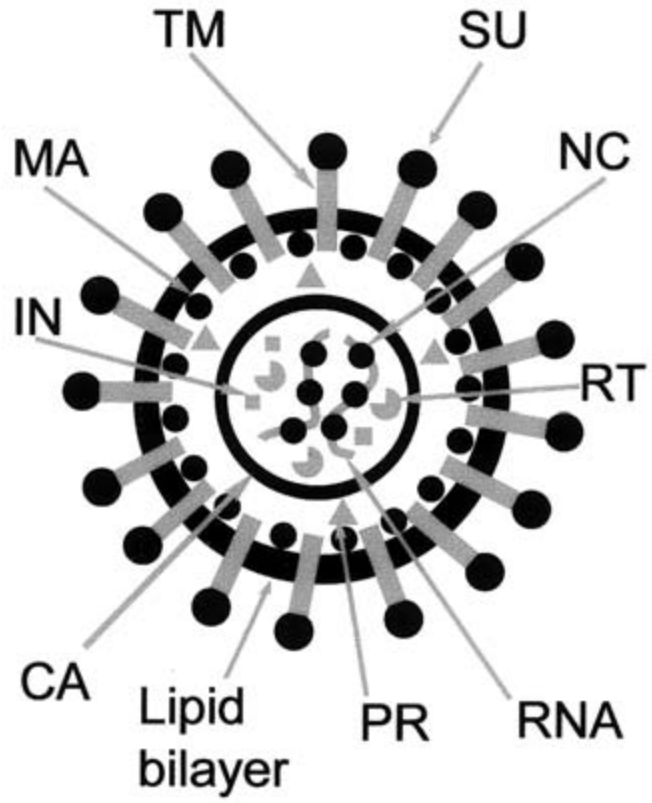
Classification

Viruses of the avian leukosis/sarcoma group are placed, in a new classification of the International Committee on Taxonomy of Viruses (ICTV), in the *Alpharetrovirus* genus of the family Retroviridae (210). Members of this family are RNA viruses characterized by the possession of the enzyme reverse transcriptase, which is necessary for the formation of a DNA provirus that is integrated in the host genome during virus replication. Under the new taxonomy, *avian leukosis virus (ALV*) is the type species of the genus (Figures 15.21 and 15.22). Other species in the genus are *Rous sarcoma virus* (RSV) and a number of replication defective viruses carrying various oncogenes.

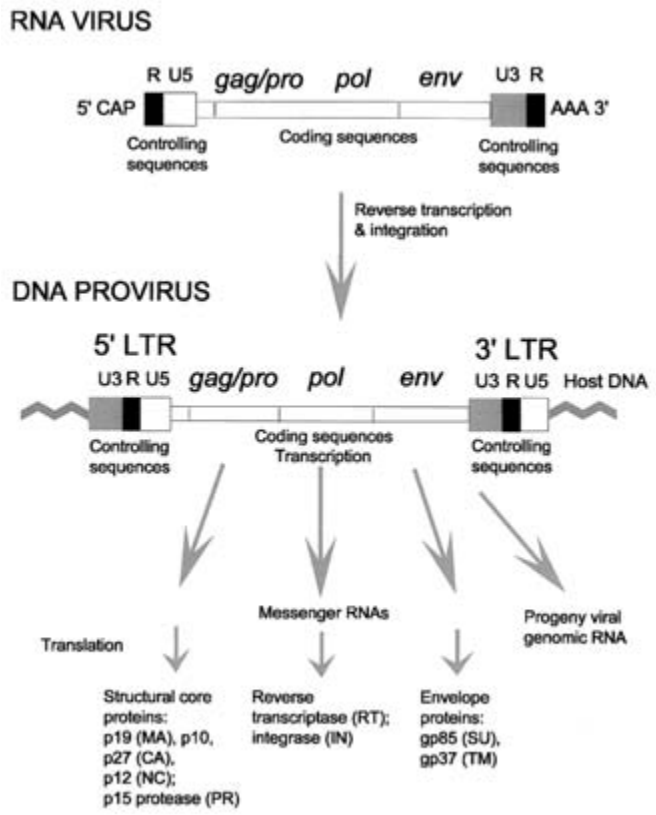
Morphology

Ultrastructure

In thin-section electron microscopy, avian leukosis/sarcoma viruses (ALSV) have an inner, centrally located electron-dense core about 35–45 nm in diameter, an intermediate membrane, and an outer membrane. This appearance typifies the C-type retroviral morphology. The overall diameter of the virus particle is 80–120 nm, with an average of 90 nm. Immature virions budding from the cell membrane can be visualized (Fig. 15.23).



15.21. Schematic diagram of avian leukosis virus particle. The viral envelope is a lipid bilayer in which the gp37 transmembrane (TM) and the gp85 surface (SU) proteins, encoded by the env gene, are inserted. Internal components encoded by the gag/pro gene are p19 matrix (MA) protein, p27 capsid (CA) protein, p12 nucleocapsid (NC) protein, and p15 protease (PR). The pol gene encodes the reverse transcriptase (RT) and p32 integrase (IN). The core of the particle contains 2 viral RNA strands.



15.22. Key features of the viral RNA and proviral DNA forms of the genome of avian leukosis virus. CAP, 5′end structure; AAA, polyadenylation of 3′end; R, repeat sequence; U5, unique 5′ end sequence; U3, unique 3′ end sequence; LTR, long terminal repeat. For other abbreviations, see Figure 15.21 and the text.

Negatively stained preparations reveal essentially spherical particles that are readily distorted under certain conditions of drying (35). Characteristic knobbed spikes about 8 nm in diameter are present on the surface of the particles and comprise the viral envelope glycoproteins. These projections can also be seen in thin sections.

Size and Density

By filtration through membranes of graded pore size, ultracentrifugation, and electron microscopy, viruses have a diameter of 80–145 nm. The value of 1.154–1.17 g/mL for the buoyant density in sucrose is characteristic for C-type retroviruses (29, 427).

Symmetry

Although the particle and core of ALSV show no obvious symmetrical features, the cores of some C-type retroviruses have icosahedral symmetry.

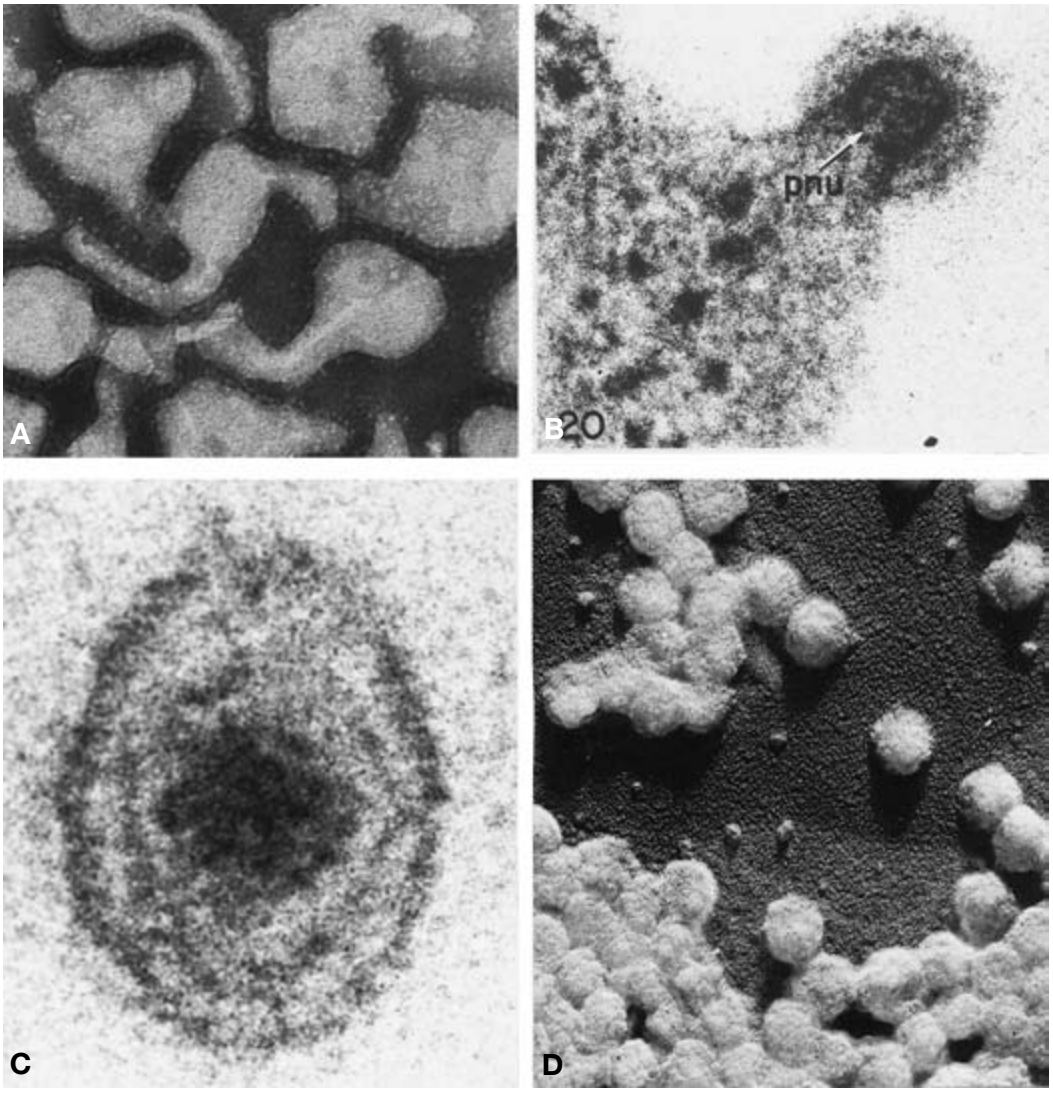
Chemical Composition

The overall composition of avian myeloblastosis virus (AMV), which has been studied extensively, is 30–35% lipid and 60–65% protein, of which 5–7% is glycoprotein, 2.2% is RNA, and small amounts of DNA are present, apparently of cellular origin (30, 33, 537).

Viral Nucleic Acids

The major class sizes of RNA sediment at 60–70S, which is the viral genome, and at 4–5S, most of which is host tRNA, are thought to be accidentally included in the virion and to play no role in viral replication. A tRNA is also associated with the 70S RNA and is a primer for the DNA polymerase during transcription of viral RNA to DNA. Small amounts of 18 and 28S RNA, viral and cellular mRNA, and DNA are also present. The 60–70S genomic RNA is a dimer and can be split into two subunits of about 34–38S, which are believed to represent the diploid genome. These subunits of genomic RNA are mRNAs, and their genes have been mapped for a number of avian retroviruses.

The sequence of the structural genes of ALV, from the 5′ end to the 3′ end of the RNA molecule, is *gag/pro-pol-env*; these genes encode, respectively, the proteins of the virion group-specific (gs) antigens and protease, RNA-dependent DNA polymerase (reverse transcriptase, or RT), and envelope glycoproteins



15.23. Ultrastructure of leukosis/sarcoma viruses. A. BAI-A of avian myeloblastosis virus (AMV), unfixed, and negatively stained with neutralized phosphotungstic acid. Peripheral fringe about particles is resolved in some places into discrete "knobs." ×150,000. B. Ultrastructure of leukosis/sarcoma virus release. Virus budding at cell membrane of a leukemic myeloblast. Surface of buds and particles peripheral to outer membrane is irregular and indistinct (pnu, dense prenucleoid). ×215,000. C. Thin section of BAI-A of AMV sedimented from plasma, fixed in osmium tetroxide, and stained with lead subacetate. Inner and outer membranes and granular character of nucleoid can be seen. Impression of granules might be derived from sectioning of filaments. Some granules appear to be hollow. ×510,000. D. Purified BAI-A of AMV fixed and shadowed with chromium. ×50,000. (Bonar and de Thé)

(Fig. 15.22). The structural genes are flanked by terminal genomic sequences with gene promoter and enhancer activities, and that, in the DNA provirus, form the long terminal repeat (LTR) regions. The viral genome is about 7.3 kb in size.

The so-called acutely transforming viruses possess additional viral oncogene sequences that initiate neoplastic transformation. Acquisition of a viral oncogene usually is accompanied by genetic defects elsewhere in the viral genome (see "Pathogenicity"). Nondefective RSV has the genetic composition *gag/pro-pol-env-src*. The additional gene, *src*, responsible for sarcomatous transformation, evidently was acquired originally from a normal cellular oncogene, cellular *src*. The inclusion of this gene is responsible for the approximately 35S subunits of RSV RNA being slightly larger than those of slowly transforming leukosis viruses. The gene cellular *src* is an example of a number of host cell genes, termed proto-oncogenes or *onc* genes, concerned with acute transformation (181, 542). Viral and cellular versions of *onc* genes, and of the specific varieties such as *src*, are distinguished by the prefixes v- and c-. Specific v-*onc* genes, with c-*onc* counterparts in normal cells, are present in other acutely transforming viruses, as listed in Table 15.6.

Slow transformation, as in LL, is caused by an indirect mechanism independent of a v-*onc* but dependent on activation of a c-*onc*, namely c-*myc* in LL, by adjacent integration of ALV provirus (303, 304). This mechanism is called insertional mutagenesis.

Viral Lipids

Viral lipids, mainly phospholipid, occur in the virion envelope and are of cellular origin. They have a bilayered structure similar to the outer cell membrane from which the virion envelope is derived (30, 48).

Viral Proteins

The nature, location, and synthesis of proteins that constitute avian retroviruses have been extensively studied (501) (Figs. 15.21 and 15.22). The virion core contains 5 non-glycosylated proteins encoded by the *gag/pro* gene: MA (matrix, p19); p10; CA (capsid, p27), which is the major gs antigen (Gag) in the core shell; NC (nucleocapsid, p12), involved in RNA processing and packaging; and PR (protease, p15), involved in cleavage of protein precursors. Other minor polypeptides have been reported.

The *pol* gene encodes the enzyme reverse transcriptase (RT) present in the core. It is a complex consisting of the b subunit (95 kD) and the a subunit (68 kD) derived from it and has RNA- and DNA-dependent polymerase and DNA:RNA hybrid-specific ribonuclease H activities. The b subunit also contains the IN domain (integrase, p32), the enzyme necessary for integration of viral DNA into the host genome.

The virion envelope contains 2 glycoproteins encoded by the *env* gene: SU (surface, gp85), the viral surface knob-like structures that determine viral envelope subgroup specificity of the ALSV; and TM (transmembrane, gp37), representing the transmembrane structure that attaches the knobs to the envelope. These 2 envelope (Env) proteins are linked to form a dimer, termed virion glycoprotein (VGP).

Enzymes and other proteins are found in virions and are considered to be cellular components incorporated during virus maturation (501). Of practical value is the presence in AMV obtained from blood of infected chickens, or from myeloblast cultures, of adenosine triphosphatase derived from the cell membrane and incorporated into the virus particle during maturation. This enzyme will dephosphorylate adenosine triphosphate, and this activity can be used for virus assay and purification. Cells without this enzyme, such as fibroblasts, release virus that is devoid of this activity.

Virus Replication

As with other retroviruses, replication of ALSV is characterized by the formation, under the direction of reverse transcriptase, of a DNA provirus that becomes linearly integrated into the host cell genome (Fig. 15.22). Subsequently, the proviral genes are transcribed into viral RNAs, which are translated to produce precursor and mature proteins that constitute the virion. Great effort has been made since the 1970s to elucidate these events, details of which have been reviewed extensively (320, 506). Only an outline of the main events is provided here.

Penetration of the Host Cell

Detailed reviews describing the recent understanding of the early ALV interactions with the host cells are available (27, 28). Although adsorption of the virion to the cell membrane is nonspecific, occurring even in cells resistant to infection, penetration of cells is dependent on the presence, in the cell membrane, of host gene-encoded receptors specific for particular virus envelope subgroups and on fusion of viral and cell membranes. Virions are taken into the cell in vacuoles and viral RNA in the nucleus within 120 minutes of attachment (141). In recent years, considerable progress has been made in understanding the nature of the receptors used by the different ALV subgroups (27). The receptor for subgroup A ALV, designated TVA, is related to the human low-density lipoprotein receptor (29, 571). Binding of the virus to the receptor for subgroup A ALV triggers a conformational change in the viral envelope glycoprotein that allows viral fusion with the cell membrane and viral entry (231). The receptors for ALV subgroups B, D, and E, designated TVB^{s3} and TVB^{s1}, resemble a receptor for cytokines of the tumor necrosis factor family (1–3, 298); the resistance to these viruses is due to a premature stop codon within this allele (296) and molecular tests to evaluate TvB haplotypes have been developed (577). The TVB receptors are functional death receptors capable of inducing death-signalling pathways leading to apoptosis (56, 297). The receptor for the subgroup C avian sarcoma and leukosis viruses, Tvc, is related to mammalian butyrophilins, members of the immunoglobulin superfamily (175). The host cell receptor used by the ALV subgroup J, which has a distinct envelope with limited homology to those of other subgroups, has recently been identified as the chicken Na(+)/H(+) exchanger type 1 (chNHE1) protein (91).

Synthesis and Integration of Viral DNA

Detailed review on the synthesis and integration of viral DNA have been provided elsewhere (58). Major stages in formation of retroviral DNA are (1) synthesis of the first (minus) strand of viral DNA by reverse transcription of viral RNA by reverse transcriptase, forming an RNA:DNA hybrid; (2) removal of RNA from the hybrid by RNase-H and formation on the template of minus-strand DNA of second (plus) strands of viral DNA, giving rise to linear DNA duplexes (these duplex molecules are detectable in cytoplasm of the cell within a few hours of infection); and (3) migration of linear DNA to the cell nucleus.

Linear viral DNA becomes linearly integrated into the host DNA under the influence of the enzyme integrase. This integration can occur at many sites, and infected cells can contain up to 20 copies of viral DNA. The proviral genes occur in the same order as their RNA copies occur in the virion, and they are flanked on either side by identical sequences of nucleotides—the long terminal repeats (LTRs) (Fig. 15.22). These are composed of repeated sequences derived from terminal regions of viral RNA and include promoter and enhancer sequences controlling transcription of viral DNA to RNA. The LTR promoters may also cause abnormal transcription of host genes usually downstream of the proviral DNA, leading to oncogenesis.

Transcription

Formation of new virions in the infected cell is the result of transcription and translation of proviral DNA, the major events being as follows: (1) Transcription of viral RNA on a template of proviral DNA occurs under the influence of a host RNA polymerase.

Table 15.6. Acutely transforming avian sarcoma and leukemia viruses classified according to viral oncogene.

Virus strain	Oncogene(s) carried	Oncogene product	Predominant neoplasm(s)	Cells transformed *in vitro*
RSV, B77, S1, S2	*src*	Nr ptk	Sarcoma	Fibroblast
FuSV, UR1, PCR II, PCR IV	*fps*	Nr ptk	Sarcoma	Fibroblast
Y73, ESV	*yes*	Nr ptk	Sarcoma	Fibroblast
UR2	*ros*	R ptk	Sarcoma	Fibroblast
RPL30	*eyk*	R ptk	Sarcoma	Fibroblast
ASV-17	*jun*	Tf	Sarcoma	Fibroblast
ASV-31	*qin*	Tf	Sarcoma	Fibroblast
AS42	*maf*	Tf	Sarcoma	Fibroblast
ASV-1	*crk*	Ap	Sarcoma	Fibroblast
AEV-ES4,	*erb*A, *erb*B	Tf, R ptk	Erythroblastosis, sarcoma	Erythroblast, fibroblast
AEV-R	*erb*A, *erb*B	Tf, R ptk	Erythroblastosis	Erythroblast
AEV-H	*erb*B	R ptk	Erythroblastosis, sarcoma	Erythroblast, fibroblast
S13	*sea*	R ptk	Erythroblastosis, sarcoma	Erythroblast, fibroblast
E26	*myb, ets*	Tf	Myeloblastosis, erythroblastosis	Myeloblast, erythroblast
AMV	*myb*	Tf	Myeloblastosis	Myeloblast
MC29	*myc*	Tf	Myelocytoma, endothelioma	Immature macrophage, fibroblast
CMII	*myc*	Tf	Myelocytoma	Immature macrophage, fibroblast
966 ALV-J	*myc*	Tf	Myelocytoma	Immature macrophage
OK10	*myc*	Tf	Endothelioma	Immature macrophage, fibroblast
MH2	*myc, mil*	Tf, S/tk	Endothelioma	Immature macrophage, fibroblast

Note: Ap 5 Adaptor protein; Nr ptk 5 Nonreceptor protein tyrosine kinase; R ptk 5 Receptor protein tyrosine kinase; S/tk 5 Serine/threonine kinase; and Tf 5 Transcription factor.

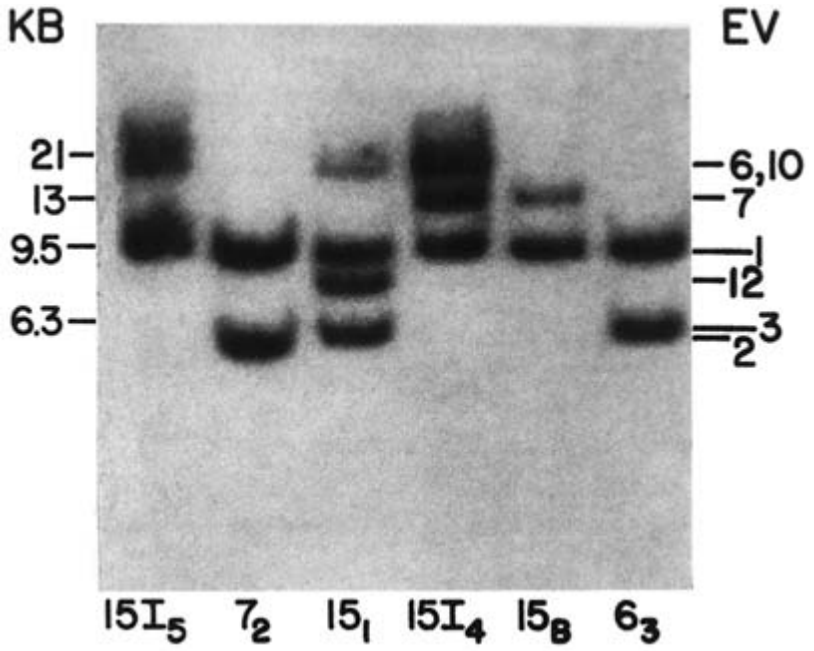
Transcription of the *pro* sequence of the *gag/pro* gene to produce protease (PR) involves a frame shift. Viral RNA molecules give rise to mRNA in association with polyribosomes, and they also serve as genomic RNA in newly formed virions. New viral RNA is detectable within 24 hours of infection. (2) mRNA species, bound to polyribosomes, are translated to form the *gag*, *pol*, and *env* gene-coded proteins that comprise the virion. The *gag/pol* gene product is a large protein precursor (180 kD) Pr180, which is cleaved to give a precursor polyprotein Pr76 (76 kD) from which virion core proteins MA (p19), CA (p27), NC (p12), PR (p15) and p10 are derived. The Pr180 polyprotein also gives rise to RT (p63 and p95) and integrase (IN, p32) enzymes. The *env* gene product is a precursor protein gPr92 (92 kD) from which the viral envelope proteins SU (gp85) and TM (gp37) are derived. Translation of *env* is from a spliced subgenomic RNA. The viral proteins localize at the plasma membrane of the cell, where crescent-shaped structures develop and virions that bud off from the cell may be visualized.

Defectiveness and Phenotypic Mixing

A number of avian retroviruses (Table 15.6) have been shown to have defective genomes and arise either spontaneously or as a result of experimental mutagenesis (255). Some viruses (certain strains of RSV and acute leukemia viruses) are defective for genes required for replication and are termed replication-defective (*rd*) mutants. They will transform cells but require the presence of a helper leukosis virus to enable them to replicate (e.g., BH-RSV and AMV lack the *env* gene, and AEV and MC29 lack the *pol* and *env* genes). Other acutely transforming viruses, such as certain strains of RSV, have lost their v-*onc* gene and ability to transform rapidly: They are called transformation defective (*td*) mutants and have an oncogenic potential similar to that of nondefective ALVs (45). *Td* and *rd* mutants are defective under all conditions (nonconditional mutants). Conditional mutants function under permissive conditions, not under nonpermissive conditions, and are exemplified by the temperature-sensitive (*ts*) mutants.

BH-RSV is the classic example of an *rd* mutant and is of practical importance in the non-producer (NP) cell activation test for ALVs (see "Diagnosis"). On single infection of chick embryo fibroblasts, the defective virus genome of BH-RSV functions to bring about replication of viral RNA, transformation of infected cells, and production of gs antigen. However, only noninfectious progeny particles are produced, which are unable to enter new host cells because of an alteration in their envelope glycoproteins. The morphologically altered cells are called NP cells. A nondefective ALV added to these cells acts as a helper virus by complementing the defective genome of BH-RSV and causes both infectious RSV and progeny ALV to be produced simultaneously. Thus, the presence of infectious RSV in the NP test denotes the presence of ALV in added test material. This RSV has envelope antigens identical to those of the helper virus, which, thus, determines infectivity and range of infectivity in genetically different cells, interference patterns among and between virus subgroups (see "Strain Classification"), and envelope antigenicity.

Stocks of *rd* mutant RSV must by their existence contain



15.24. Endogenous viral (ev) loci detected in 6 inbred lines of white leghorns by restriction fragment polymorphisms generated after Sac-1 endonuclease digestion of red blood cell DNA and hybridized to ^{32}P-labeled RAV-2 genomic sequences. (464)

helper viruses; these originally were referred to as Rous-associated viruses (RAVs). Infectious RSVs formed in these circumstances are called pseudotypes, and their designation includes the helper virus when this is identified (e.g., BH-RSV(RAV-1) when RAV-1 strain ALV helper virus is used). This phenomenon is an example of the phenotyping mixing (PM) (47) that occurs readily when 2 related viruses infect the same cell, and in which virions with the genome of one virus may possess envelope and other structural proteins of the other parent, or both. The phenomenon of PM is also used in the PM test for detection of ALV (see "Diagnosis"). Genetic recombination, in which exchanges of genes between 2 viruses (and consequent stable phenotypic changes) occur, are well recognized and must be distinguished from PM (552, 569). Use of defective strains of RSV enables tailor-made RSV to be produced with envelope properties of the helper ALV. Determinations of host range, interference pattern, and neutralization can be performed more easily with the appropriate RSV pseudotype than with the ALV, because the former can be readily quantified in cell culture.

These factors notwithstanding, infectious RSV may be generated with BH-RSV and other *rd* mutants following solitary infections in the absence of added helper viruses in certain types of chicken cells that carry endogenous ALV genomes (see "Endogenous Leukosis Viruses"). Such RSV has the subgroup E host range of the endogenous virus, however, and may be distinguished from helper viruses of other subgroups when NP tests are conducted.

Phenotypic mixing may also occur between unrelated viruses, such as vesicular stomatitis virus (VSV) and ALVs (550) or between reticuloendotheliosis virus (REV) and RSV (450). The VSV with ALV viral envelope may be used in rapid interference, host range, and neutralization tests because it is rapidly cytopathic.

Endogenous Leukosis Viruses

ALVs that are transmitted as infectious virus particles are termed exogenous viruses. The normal chicken genome also contains several classes or families of avian retrovirus-like elements (117) that are transmitted genetically and are termed endogenous viruses. These include the endogenous viral (*ev*) loci, recognized some 30 years ago, and the more recently discovered moderately repetitive elements EAV (endogenous avian virus) (53, 169) and ART-CH (avian retrotransposon from chicken genome) (50, 357, 440) and the highly repetitive elements CR1 (chicken repeat 1) elements (499). Endogenous retroviruses in chickens are examples of the numerous retroelements present in eukaryotes. Certain retroelements are believed to represent stages in the evolution of retroviruses from cellular movable genetic elements (transposons); whereas others are thought to be degenerate proviral forms of exogenous retroviruses that have lost the ability to produce infectious virus due to mutations. The significance of these elements is currently the subject of much research.

The genetic sequences of the *ev* loci are related to subgroup E ALVs and are present as either complete or defective genomes in almost all normal chickens (116, 119, 424, 464) (Fig. 15.24). The chromosomal locations of a number of *ev* loci have been determined (509) and the availability of the chicken genome sequence (108) has enabled the identification of their genomic locations. They occur in somatic and germ line cells and are transmitted genetically in a Mendelian fashion to their progeny by both sexes (8, 127). At least 29 *ev* loci have been identified (246, 464) and locus-specific PCR tests for their identification have been described (39). Each chicken has been reported to carry on average about 5 *ev* loci (433). The phenotypic expressions of these loci vary, depending on the viral genes present and on poorly understood control mechanisms (Tables 15.7 and 15.8). When the complete endogenous viral genome is present, the cell may produce subgroup

Table 15.7. Phenotypic expression of representative endogenous avian leukosis viral (*ev*) genes in normal chicken cells.

Phenotype	Symbol	*ev* locus
No detectable viral product	gs^2chf^2	1, 4, 5
Expression of subgroup E envelope antigen	gs^2chf^1	9
Coordinate expression of group-specific and envelope antigens	gs^1chf^1	3
Spontaneous production of subgroup E virus	$V\text{-}E^1$	2

Source: Adapted from Smith (464)

Table 15.8. Phenotypes of endogenous avian leukosis (*ev*) genes in inbred and commercial lines of white leghorn chickens.

ev	Phenotype	Line or Source[a]
1	gs^2chf^2	Most lines
2	$V\text{-}E^1$	RPRL72
3	gs^1chf^1	RPRL63
4	gs^2chf^2	SPAFAS
5	gs^2chf^2	SPAFAS
6	gs^2chf^1	RPRL151
7	$V\text{-}E^1$	RPRL15B
8	gs^2chf^2	K18
9	gs^2chf^1	K18
10	$V\text{-}E^1$	RPRL 15I4
11	$V\text{-}E^1$	RPRL 15I4
12	$V\text{-}E^1$	RPRL 151
14	$V\text{-}E^1$	H & N
15 (C)	None	K28 3 K16
16 (D)	None	K28 3 K16
17	gs^2chf^2	RC-P
18	$V\text{-}E^1$	RI
19	$V\text{-}E^1(?)^b$	RW
20	$V\text{-}E^1(?)^b$	RW
21	$V\text{-}E^1$	Hyline FP

Note: *Ev*13 is associated with the gs^2chf^2 phenotype, but restriction fragments have not been characterized.
[a] Not exclusive to line or source. K, Kimber; R, Reaseheath; H & N, Heisdorf and Nelson; for references see Smith (464).
[b] The presence of 5 *ev* loci in Reaseheath line w birds precludes definitive assignment with the $V\text{-}E^1$ phenotype. Definitive association requires further segregation of *ev* genes. Hyline FP birds also carry *ev*1, *ev*3, and *ev*6.

E ALV, either spontaneously or after induction by chemicals such as bromo-deoxyuridine (BUDR). When the endogenous virus is incomplete (defective), genes present may be expressed phenotypically in the cells, but infectious virus is not produced. This is because of the absence of the complete set of genes needed for infectious virion production (e.g., the defective *ev*3 locus possesses the *gag* and *env* genes of subgroup E ALV, and cells carrying this locus contain gs antigen and subgroup E viral envelope glycoproteins). However, the locus has a genetic deletion around the *gag-pol* junction, and infectious virions are not formed. The presence of *ev* genes in such cells is responsible for their positive reactions in the enzyme-linked immunosorbent assay (ELISA) test, the complement-fixation test for avian leukosis viruses (COFAL), and the chick helper factor (chf) test (see "Diagnosis"). In the chf test, genetic defectiveness in the envelope of BH-RSV is complemented by endogenous envelope proteins, resulting in an infectious form of RSV with subgroup E host range and other properties. Genetic characteristics of *ev* loci are described in detail (117, 464, 594, 551). The expression of endogenous *ev* genes is responsible for a dominant form of genetic resistance of chicken cells to infection by subgroup E ALV from a block of virus receptors by envelope protein (392, 425). Transmission of *ev* genes from parent to offspring has been called genetic transmission of ALV (548), distinguishing it from the vertical (congenital) and horizontal (contact) transmission of viruses in an infectious state. However, fully expressed infectious endogenous virus may sometimes also be transmitted vertically and horizontally (475). Related *ev* loci occur in several species of fowl, other than domestic chickens, including red jungle fowl and some strains of pheasant, partridges, and grouse, but the distribution does not support a phylogenetic relationship (219, 230). Rather, it is considered that the ALV genomes have become incorporated at various loci relatively recently in the history of *Gallus* and independent of integration in other genera. The presence of ALSV *gag* genes in 26 species of galliform birds recently was reported (153, 154). Evidence of congruence of ALSV and host phylogenies exists but also of horizontal ALSV transmission between hosts of different phylogenies. It is not known whether endogenous viruses arise from exogenous viruses of other subgroups, from which they differ genomically at the *env* gene and the LTR region, or vice versa.

Subgroup E ALV, typified by the RAV-0 strain of ALV, has little or no oncogenicity (346), apparently because of the weak promoter activity of the LTR. Persistence of these viral loci suggests that birds carrying them are not at a great disadvantage, and they may be beneficial. Thus, Crittenden *et al.* have shown that the presence of *ev*2 or *ev*3 protects birds from a unique non-neoplastic syndrome caused by infection with an exogenous subgroup A ALV (122, 133). Endogenous viruses will have either beneficial or detrimental effects, perhaps by their induction of immunity or tolerance to tumor virus antigens, depending on when they are expressed. Embryonic infection with endogenous ALV, RAV-0, caused more persistent viremia and more neoplasms following infection with exogenous ALV, apparently due to tolerant depression of specific humoral immunity (125). Similarly, subgroup E recombinants of endogenous and exogenous viruses have been reported to be capable of inducing neoplasm (123). Endogenous viruses of the *ev* family are not essential, because it has been possible to produce chickens free of *ev* genes (7). A line of such chickens has been produced, designated line 0 (121), that is of value in research studies and certain diagnostic tests in which birds or cells free from *ev* loci are needed. Other chickens lacking *ev* loci have been recognized (117), but the great majority have these endogenous sequences. More re-

cently, methods of developing commercial chicken strains free of endogenous retroviruses have been described (15).

Of particular importance is the *ev*21 locus, which is linked tightly in white leghorn stock to the dominant sex-linked gene, *K*, on the Z chromosome (16), which regulates slow feathering. It is possible that insertion of the *ev*21 sequence into a feather growth locus is responsible for the slow feathering mutation (117). Some breeders producing feather-sexed crosses have reported reduced egg production and higher leukosis mortality associated with an increased incidence of viremia with exogenous ALV in fast-feathering female progeny from dams carrying the *K* gene. The *ev*21 gene is expressed as an infectious endogenous ALV, EV21, in the dam, which is transmitted congenitally to the progeny, inducing immunological tolerance and, consequently, increased susceptibility to infection by exogenous ALV (16, 252, 466, 468). Strategies to overcome the *ev*21 locus effect have been studied (470, 471, 473).

The biologic functions, if any, of the other endogenous elements described, namely EAV, ART-CH, and CR1, remain to be determined. Members of the EAV family are not expressed as infectious virus, but RT activity can be expressed and has been found in live virus vaccines (266, 267, 423, 519, 556). A member of the EAV family, EAV-HP (also termed ev/J), is believed to be the origin of the *env* gene of subgroup J ALV (21, 41, 42, 149, 438, 440, 478). The 5′ sequences of ART-CH and EAV-HP are almost identical (442). Embryonic expression of EAV-HP env has been suggested to be associated with the induction of immunological tolerance, a feature observed in a significant proportion of meat-type chickens infected with ALV-J. In support of this hypothesis, it was recently demonstrated that EAV-HP loci are still segregating within the chicken population (442). Recently the strongest evidence of the role of EAV-HP in the emergence of ALV-J by recombination was obtained from the identification of an intact chicken EAV-HP locus showing a uniquely close relationship to the ALV-J prototype clone HPRS-103 env region (441).

CR1 elements are non-LTR-containing retroelements (retroposons) possessing RT sequences. They are very numerous and are considered ancient and primitive sequences, preceding the evolution of birds, and are not functionally expressed (178, 318, 527, 546, 558).

Susceptibility to Chemical and Physical Agents

Lipid Solvents and Detergents

Avian retroviruses have a high lipid content in the envelope, and their infectivity is abolished by ethyl ether (217). The detergent sodium dodecyl sulfate disrupts the virions and releases RNA and core proteins (427).

Thermal Inactivation

The half-life of various ALSVs at 37°C varies from 100–540 minutes (average, around 260 minutes), depending on the medium in which the virus is suspended, the tissue of origin, and the virus strain. ALSVs are inactivated rapidly at high temperatures; the half-life for RSV at 50°C is 8.5 minutes and at 60°C is 0.7 minutes (163).

Thermal lability of infectivity of these viruses is a critical factor in storage. Even at −15°C, the half-life of AMV is less than 1 week (172); it is only at temperatures below −60°C that avian retroviruses can be stored for several years without loss of infectivity (61). Freezing and thawing degrade the virus, and the gs antigen is released.

pH Stability

The stability of viruses of this group changes little between pH 5 and 9; outside this range, inactivation rates are markedly increased.

Ultraviolet Irradiation

RSV and field strains of ALV are relatively resistant to exposure to ultraviolet light (217, 435).

Strain Classification

Antigenicity

ALSVs that occur in chickens have been divided into 6 envelope subgroups, A, B, C, D, E, and J, on the basis of differences in their viral envelope glycoproteins, which determine antigenicity, viral interference patterns with members of the same and different subgroups, and host range in chicken embryo fibroblasts of different phenotypes (105, 555). The other subgroups, F, G, H, and I, represent endogenous ALVs occurring in pheasants, partridge, and quail (374).

Viral interference patterns (Table 15.9) and host range patterns (Tables 15.10 and 15.11) are the most reliable methods for subgroup classification. Antigenicity, as determined by the production of neutralizing antibodies or neutralization by known subgroup-specific antibodies, can also be used for strain classification, but is less dependable. Viruses within a subgroup usually cross-neutralize to varying extents, but with the exception of partial cross-neutralization between subgroup B and D viruses, viruses of different subgroups do not. However, antisera against particular isolates of subgroup J virus do not always cross-neutralize other J isolates, or they may show one-way cross-

Table 15.9. Interference patterns between ALV and RSV of subgroups A–E and J.

Subgroup of interfering ALV	Subgroup of challenge RSV					
	A	B	C	D	E	J
A	1	2	2	2	2	2
B	2	1	2	1	1	2
C	2	2	1	2	2	2
D	2	2	2	1	2	2
E	2	2	2	2	1	2
J	2	2	2	2	2	1

Note: Susceptible avian embryo fibroblast cultures are infected with ALV of each subgroup and challenged several days later with RSV of each subgroup. Reduction in RSV foci in infected cultures compared with uninfected controls is indicative of viral interference. 1, interference; 2, no interference.

Table 15.10. Examples of host range of subgroup A–E and J avian leukosis/sarcoma viruses in chicken embryo cells of different phenotypes.

Phenotype of cells	Examples (chicken or cell lines)	Subgroup of virus					
		A	B	C	D	E	J
C/0	15B1	S	S	S	S	S	S
C/AE	C, alv6	R	S	S	S	R	S
C/A,B,D,E	7_2	R	R	S	R	R	S
C/E	0, 15I, BrL	S	S	S	S	R	S
C/EJ	DF-1/J[a]	S	S	S	S	R	R

Note: S, susceptible; R, resistant. The cell phenotype designation denotes chicken (C) cells resistant to (/) the specified subgroup (0, no subgroup; AE, subgroups A and E; etc.).
[a] Cell line, Hunt *et al.* (265).

Table 15.11. Host range of different subgroups of Rous sarcoma virus (RSV) in embryo fibroblasts of various avian species.

Avian species	Subgroup of RSV					
	A	B	C	D	E	J
Red jungle fowl	S	S	R	R	R	S
Common pheasant	S	R	R	R	S	R
Japanese quail	S	R	R	R	S	R
Guinea fowl	S	S	S	S	S	R
Turkey	S	S	S	S	S	S
Peking duck	R	R	S	R	R	R
Goose	R	R	S	R	R	R

Note: Embryo fibroblast cultures from avian species are challenged with RSV and susceptibility to RSV focus formation determined. S, susceptible; R, resistant. Data from Payne *et al.* (389).

neutralization (193, 204, 206, 211, 532). In general, antiserums raised against a particular strain of virus tend to neutralize the homologous virus more strongly than heterologous viruses of the same subgroup (101). These findings indicate the occurrence of varying antigenic epitopes within subgroups. Subgroup B viruses appear to be more heterogeneous than those of subgroup A, and subgroup J viruses are especially variable (528, 532, 543).

Molecular Characteristics

Sequence analysis of the gp85 encoding sequences of the *env* genes of ALVs of subgroups A–E have identified 2 hypervariable regions, hr1 and hr2, and 3 less variable regions, vr1, vr2 and vr3, in which differences between the subgroups are present (51, 52, 162). Studies of recombinants indicated that hr1 and hr2, and to a lesser extent vr3, play the major role in determining receptor tropism (161). However, the exact locations and nature of the differences that determine host range and antigenicity have not yet been identified. The gp85 sequence of the *env* gene of subgroup J ALVs differs more extensively from those of the other 5 subgroups, notably at hr1, hr2, vr2, and vr 3, and to a lesser extent also between these regions (21, 22). Different subgroup J isolates also vary at particular hypervariable regions of gp85 (42, 461, 462, 532, 543). Primers from the variable regions of the *env* gene of subgroups A–E and J have been used in PCR tests to identify subgroups of ALV isolates (225, 289, 477, 573, 575). These tests have the potential to be used in strain classification, although more extensive studies are needed of multiple isolates within subgroups to evaluate their specificity fully.

Pathogenicity

Numerous strains of ALSVs exist, many of which were isolated from naturally occurring or experimentally induced neoplasms over many years. Some of these have been designated viral species under the new ICTV taxonomy of avian retroviruses (210). Many induce a predominant type of neoplasm and can be named accordingly; e.g., lymphoid leukosis virus (LLV), although ALV is most commonly used rather than LLV; avian erythroblastosis virus (AEV); avian myeloblastosis virus (AMV); and avian sarcoma virus (ASV) (Table 15.12). Commonly, however, the virus strains induce other neoplasms in addition to the predominant one, and the tumor spectrum can be wide. The oncogenic spectrum tends to be characteristic of a particular virus strain, but often overlaps with other strains. Thus, the RPL12 strain of ALV induces lymphoid leukosis, erythroblastosis, osteopetrosis, hemangiomas, and sarcomas; the BAI A strain of AMV induces myeloblastosis, lymphoid leukosis, osteopetrosis, nephroblastomas, sarcomas, hemangiomas, thecomas, granulosa cell tumors, and epitheliomas (34).

The spectra of tumors induced have several explanations. Some strains of virus consist of mixtures of viruses either fortuitously or because some may require coinfecting helper viruses for replication (see later discussion). However, clone-purified strains of ALV can cause a variety of neoplasms in addition to lymphoid leukosis, including erythroblastosis, osteopetrosis, and nephroblastomas (415), and clone-purified AEV can induce both erythroblastosis and sarcomas, irrespective of the helper virus (244). The relatively high mutation rate of retroviruses no doubt contributes to strain variability. The tumor spectrum of a virus strain can also be modified in passage experiments according to the type of neoplasm used to harvest virus.

Virus dose is an important factor in determining tumors induced. Thus, high doses of strain RPL12 of ALV mainly cause erythroblastosis; whereas low doses cause lymphoid leukosis (73). Factors that influence effective dose, including route of inoculation and age and genotype of the host, also influence the oncogenic spectrum.

Strains of ALSV can also be placed into two major classes in respect of rapidity of induction of tumors:

1. *Acutely transforming viruses.* These viruses can induce neoplastic transformation, *in vivo* or *in vitro*, within a few days or weeks. They cause various types of acute leukemia (leuko-

sis) or solid tumors (usually sarcomas) (181, 183, 243, 303, 342). The acutely transforming viruses are those that carry viral oncogenes in their genome (Table 15.6). Details of viral oncogenes and the biochemical functions of their products have been reviewed elsewhere (105, 303, 535). All avian acute leukemia viruses are genetically defective and require a helper leukosis virus to complement them and enable replication. The acutely transforming viruses are replication-defective (*rd*) mutants, lacking in genes required for replication. Some sarcoma viruses (e.g., Bryan high-titer strain of RSV [BH-RSV]) are also genetically defective and require a helper virus for replication.

2. *Slowly transforming viruses.* These ALVs do not carry viral oncogenes. They induce tumors by a "promoter insertion" or a related mechanism that activates a cellular oncogene to bring about neoplastic transformation and development of tumors over many weeks or months (105, 183, 221, 303).

Nomenclature

A variety of conventions, which reflect the classification methods outlined previously, are used in designating ALSVs; many of these are illustrated in Table 15.12. They are given a full and an abbreviated designation based on the predominant neoplasm they induce, with an affix to indicate their origin with an individual (e.g., Rous sarcoma virus [RSV]) or a location (e.g., Regional Poultry Research Laboratory isolate 12 [RPL12 of ALV). Substrains of RSV are designated according to individuals who worked with them (e.g., Bryan's high-titer strain [BH-RSV]) or to location (e.g., Prague [PR-RSV]). Subgroups (e.g., subgroup A) may be designated also: PR-RSV-A. The general terms *avian leukosis* (or *leukemia*) *virus* (ALV) and *avian sarcoma virus* (ASV) are used widely to designate members of the group.

Helper viruses isolated from stocks of defective viruses are named, for example, as Rous-associated virus (RAV) or myeloblastosis-associated virus (MAV), and isolates are numbered (RAV-1, MAV-1, etc.). Where a helper virus is used for replication of a defective virus, this is indicated. Thus, BH-RSV grown with RAV-1 as a helper is designated BH-RSV(RAV-1). Endogenous ALV is abbreviated EV (e.g., EV21). Strains of ALV that act as resistance-inducing factors (see "Diagnosis") were designated RIFs, but this term is now rarely used. Further details of the origins of the abbreviations (374) are given in Table 15.12.

Laboratory Host Systems

Chick Inoculation

Rous sarcoma and other sarcoma viruses produce tumors when injected by the subcutaneous (SC), intramuscular (IM), or intra-abdominal (IA) routes and at times by contact with inoculated chickens. Subcutaneous injection into the wing web or IM injection can be used for sarcoma virus isolation and propagation (340, 416), and SC inoculation has been used for TD_{50} assays of stocks of RSV (60). Following wing-web injection of high doses of RSV, tumors are first palpable at about 3 days. In susceptible chickens, these may grow rapidly, ulcerate, and metastasize; in resistant chickens, the sarcomas may regress. Extensive reviews on assay methods and interpretation of results are available (60).

ALVs may be inoculated IA or IV into day-old susceptible chicks to obtain a tumor response. Burmester and Gentry (72) inoculated strain RPL12 of ALV IA into day-old line 15I chicks and obtained a tumor response in 200–270 days. This procedure was used for initial isolation of virus from field cases (71). Time required for quantitative assay of certain strains passaged in the laboratory was shortened to 63 days by using the less sensitive erythroblastosis response (62). In these transmission experiments, all sources of virus that cause LL also caused erythroblastosis. Osteopetrosis, hemangiomas, and fibrosarcomas were also observed in chickens of certain strains and passages (70, 73).

AMV can be titrated in susceptible chicks by IV inoculation at 1–3 days of age (170, 171). AMV in chicken plasma can be assayed by its adenosine triphosphatase activity, a method useful for routine and large-scale studies (38).

Osteopetrosis-inducing activity of virus strains can be examined by IV or IM inoculation of day-old chicks (70, 260). Guinea fowl are particularly susceptible to osteopetrosis induced by MAV-2(O) (290, 291, 293, 294).

Embryo Inoculation

When RSV and other sarcoma viruses are inoculated onto the chorioallantoic membrane (CAM) of 11-day-old susceptible embryos, tumor pocks develop (Fig. 15.25), which can be counted 8 days later and are related linearly to virus dose (167). This technique is also useful for detecting genetic resistance to infection.

ALVs have been quantitated by IV inoculation into 11-day-old susceptible chicken embryos. Depending on the virus, within 2 weeks of hatching, a high incidence of neoplasms (mainly erythroblastosis) can occur, although hemorrhages and solid tumors can develop including fibrosarcomas, endotheliomas, nephroblastomas, and chondromas. When chicks are held for a postinoculation period of 46 days, responses are higher by 1–2 $\log_{10}$ dilutions than those following chicken inoculation. Most chickens that survive the acute neoplasms develop LL after 100 days postinoculation (400).

AMV produced a myeloblastosis response within a few weeks when injected by IV into susceptible embryos (23, 24, 25). When the HPRS-103 strain of subgroup J ALV was inoculated by IV into 11-day-old embryos, first death from tumor (myelocytoma) was not until 9 weeks of age, and median tumor mortality was at 20 weeks (383).

Cell Culture

RSV and other sarcoma viruses induce rapid neoplastic transformation of cells when inoculated onto monolayer cultures of chicken embryo fibroblasts (414). The transformed cells proliferate to produce within a few days discrete colonies or foci of transformed cells (Fig. 15.26), which under agar can be used for quantitative assay of virus (508). Such cell culture methods are preferred for virus assay.

Most leukosis viruses replicate in fibroblast cultures without producing any obvious cytopathic effect. Their presence can be detected by a variety of tests (see "Diagnosis"). ALVs of subgroups B and D may induce cytopathic plaques that may be used for virus assay (242). The cytopathic effect of these 2 subgroups

Table 15.12. Laboratory strains of avian leukosis/sarcoma viruses of the chicken classified according to predominant neoplasm induced and virus envelope subgroup.

	Virus class according to envelope subgroup						
Virus class according to neoplasm	**A**	**B**	**C**	**D**	**E**	**J**	**No subgroup (defective virus)[a]**
Lymphoid leukosis virus (LLV)	RAV-1 RIF-1 MAV-1 RPL12 HPRS-F42	RAV-2 RAV-6 MAV-2	RAV-7 RAV-49	RAV-50 CZAV	RAV-60		
Avian erythroblastosis virus (AEV)							AEV-ES4 AEV-R AEV-H
Avian myeloblastosis virus (AMV)							AMV-BAI-A
Avian sarcoma virus (ASV)	SR-RSV-A PR-RSV-A EH-RSV RSV29	SR-RSV-B PR-RSV-B HA-RSV	B77 PR-RSV-C	SR-RSV-D CZ-RSV	SR-RSV-E PR-RSV-E		BH-RSV BS-RSV FuSV PRCII PRCIV ESV Y73 UR1 UR2 S1 S2
Myelocytoma and endothelioma viruses						HPRS-103 ADOL-Hc1	MC29 966 MH2 CMII OK10
Endogenous virus (EV) (no neoplasm)					EV21 ILV		RAV-0

[a]Defective viruses have the envelope subgroup of their helper virus.

is explained by their use of the death receptor of the tumor necrosis factor receptor family (56, 99, 151, 152). Morphological alterations have also been reported after prolonged passage of ALV-infected fibroblasts (84).

Acutely transforming ALVs will transform hematopoietic cells *in vitro* (342). Yolk sac and bone marrow cells in culture are transformed to neoplastic myeloblasts on infection with AMV (343), and bone marrow cells transform to erythroblasts with AEV (241). Transformation of hematopoietic cells by MH2, MV29, and OK10 viruses was observed by Graf and Beug (243). Such acutely transforming ALVs have been used extensively to study avian hematopoietic cell lineages and differentiation (43, 330, 331). Acutely transforming variants of subgroup J ALV can also transform bone marrow cells and blood monocytes *in vitro* (95, 384). *In vitro* transformation of B-lymphocytes by nondefective ALV has not been reported, and bone marrow cultures were not transformed by nondefective subgroup J ALV (384).

Tumor cell lines derived from tumors induced *in vivo* by the ALVs have been developed, including LL (360, 459), myeloblastosis (311), erythroblastosis (241), and myelocytoma (312).

The properties of ALSVs in cell culture are described in more detail under "Diagnosis."

Pathogenicity

As discussed previously (see "Strain Classification"), strains of ALVs may produce more than one type of neoplasm, and the oncogenic spectrum of each strain tends to be characteristic but often overlaps with responses to other strains. Viral factors including the origin and dose, and host factors such as route of inoculation, age, genotype, and sex influence the oncogenic patterns of different virus strains.

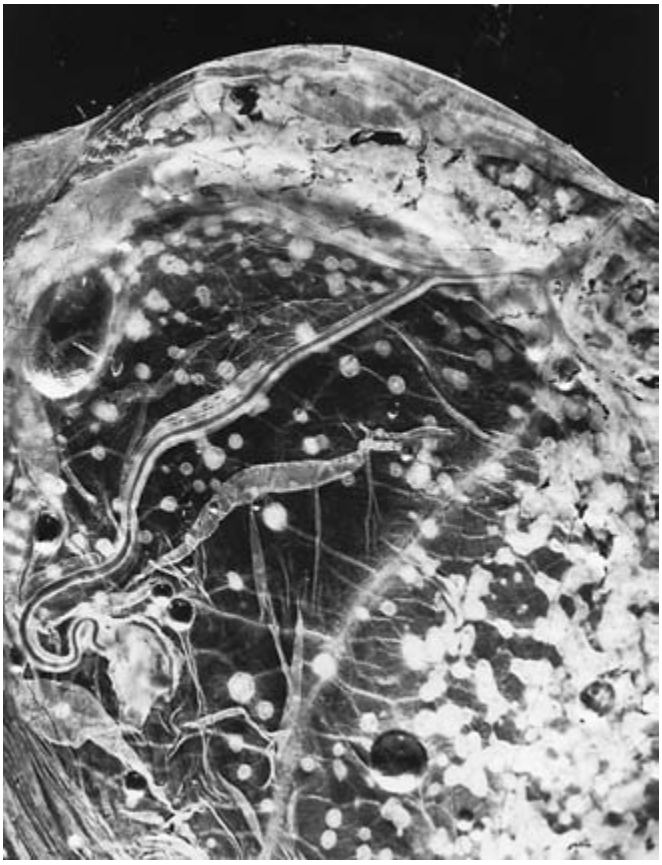
Origin of Virus

Differences in tumor spectrum may be seen in virus strains newly isolated from the field, as exemplified by tumor spectrums of RPL26, RPL27, and RPL28 isolates of ALV (215). Within a par-

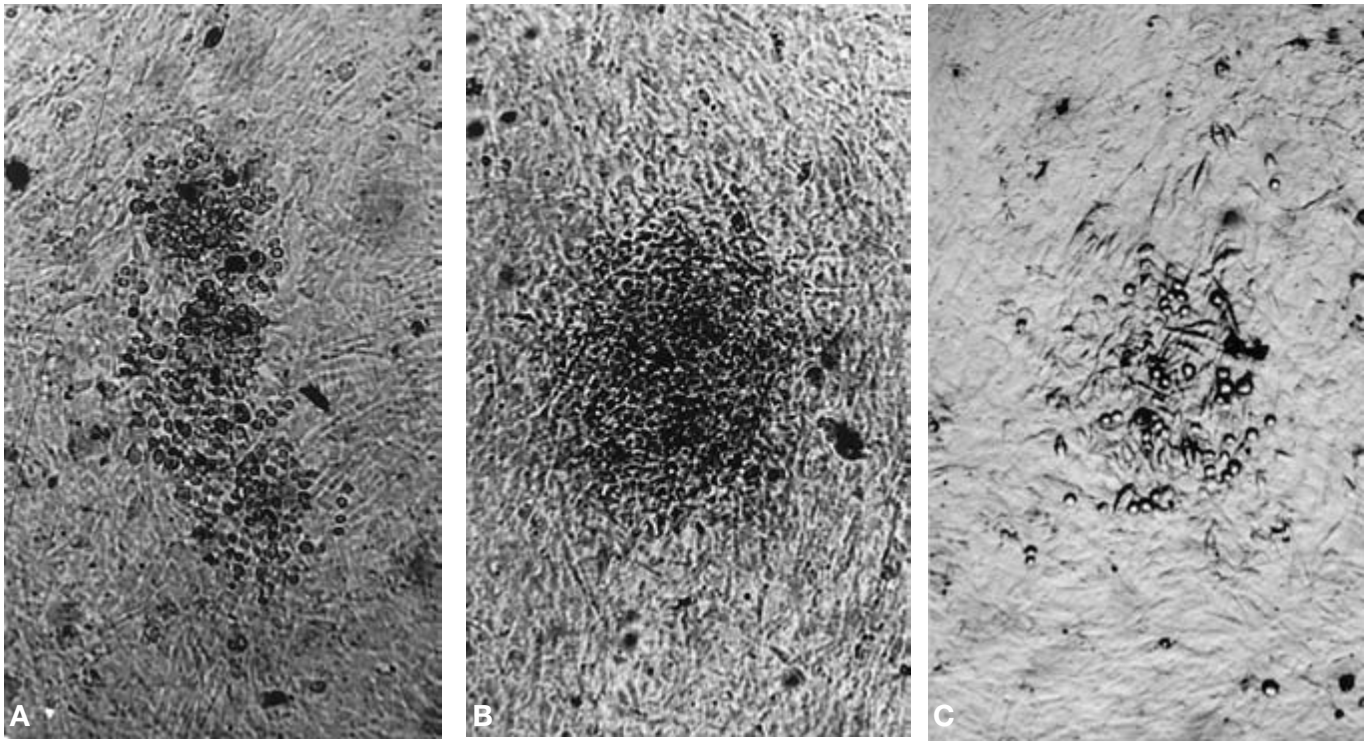
ticular strain, differences may be obtained that depend on the type of neoplasm used for virus isolation. Fredrickson *et al.* (215, 216) observed that serial transfer from donors with LL resulted primarily in LL; whereas virus from donors with erythroblastosis predominantly caused erythroblastosis. In another instance, selection of virus donors with hemangiomas resulted in a larger proportion of birds with this tumor than in a previous passage (73). In some situations, such events may be due to a virus-dose effect, but in other instances, an acutely transforming ALV with a transduced oncogene may have been generated (257).

Virus Subgroups

Usually no relationship has been observed between virus subgroup and oncogenicity except for endogenous E subgroup ALVs, such as RAV-0, which have little or no oncogenicity (346). However, the low oncogenicity of RAV-0 is believed to be related to the weak promoter activity of the subgroup E LTR and not to the *env* gene. Subgroup J ALV induced myelocytomatosis (383), and the *env* as well as other elements are thought to be associated with the unique oncogenicity (95, 96, 97, 322, 323).



15.25. Pocks induced by BH-RSV on the chorioallantoic membrane (CAM) of a chicken embryo. (Piraino)



15.26. Foci induced by Rous sarcoma virus (RSV) in cell culture. A. Unstained focus of transformed spherical, refractile chicken embryo cells infected 6 days previously with Bryan's standard strain of RSV. ×100. B. Unstained focus of transformed, polygonal, opaque Rous sarcoma cells infected 6 days previously with Bryan's high-titer strain. ×100. C. Unstained focus of transformed round and fusiform cells infected 6 days previously with Popken's preparation of RSV. ×100.

Virus Dose

High doses of RPL12 ALV mainly induced erythroblastosis; whereas doses close to the endpoint predominantly induced LL (73). Sarcomas, endotheliomas, and hemorrhages were also more common with high virus doses. Occurrence of osteopetrosis showed no dependency on dose (216).

Route of Inoculation

Responses obtained after virus administration by less efficient portals of entry into the host apparently reflect the decreased effective dose. Thus, exposure of susceptible birds by contact with birds inoculated with a high dose of strain RPL12 of ALV resulted in a LL response similar to that expected with 1/1000 of the inoculated dose (73). Intramuscular inoculation of strain RPL26 of ALV favored sarcoma induction; whereas IV inoculation mainly produced erythroblastosis and hemorrhages (216). These differences may reflect variations in amounts of virus that reach the target cells by different routes.

Age of Host

In general, resistance of birds to the development of neoplasms of all types increases with age, the rate varying with route of inoculation. Resistance increases rapidly between 1 and 21 days of age with oral or nasal administration but relatively slowly when virus is inoculated intravenously (70). Types of tumors produced also reflect the decreased effective dose (73). However, the incidence of some tumors decreases more rapidly than expected from the dose effect alone. For example, certain preparations of strain RPL12 ALV given via IV at 1 day of age caused a high incidence of osteopetrosis; the proportion of chickens inoculated at 3 weeks of age and developing osteopetrosis was only one-tenth that of chickens inoculated at 1 day of age (70). Induction of tolerant viremia at 1 day may be a factor.

Genotype and Sex of Host

The genetic constitution of the host has a strong influence on response to ALSVs (see "Pathobiology and Epidemiology"). Females are more susceptible to LL than males. Castration increases the incidence of disease, and treatment with testosterone increases resistance of males and capons (74). These effects are probably a consequence of hormonal effects influencing regression and, hence, target cell numbers in the bursa of Fabricius.

Pathobiology and Epidemiology

Incidence and Distribution

ALVs are still considered ubiquitous in commercial chickens, notwithstanding the eradication programs instituted by many primary breeding companies. With few exceptions, infection occurs in all chicken flocks; by sexual maturity, most flocks and most birds within a flock are already exposed. Nevertheless, the incidence of subgroup A ALV-induced LL, the most common neoplasm observed in infected flocks, is usually low, in the order of 1 or 2%, although losses of up to 20% can occur.

Incidence of Disease

Although sporadic cases of ALV-induced neoplasia occur in most flocks, it is only occasionally that even the most common neoplasm, LL, produces heavy losses (196, 204, 380, 394). De Boer (144, 146) reported LL mortality in the Netherlands as 2.18% of 11,220 white layers and 0.57% of 7920 brown layers, recorded in random sample tests from 1973–1979. The incidence of LL in chickens may be reduced by the widespread occurrence of infectious bursal disease virus (137, 410, 412). Conversely, serotype 2 MDV was found to enhance the development of LL in certain lines of chickens following exposure to ALV after hatch (19, 195, 198, 207). Also, an increased frequency of spontaneous bursal lymphoma (139) in white leghorn chickens inoculated with serotype 2 MDV has been reported (444) . Molecular and *in situ* hybridization analysis of the bursa from chickens co-infected with ALV and serotype 2 MDV proved that MDV was closely associated with transformed, but not with nontransformed, bursa cells (223, 326). *In vitro* studies also showed that serotype 2 MDV can increase ALV as well as RSV gene expression (26, 408, 513).

Under field conditions, erythroblastosis occurs less frequently than LL (409). However, a rare epizootic of erythroblastosis has been reported in 5-week-old birds (251). Very few reports have been made of natural occurrence of myeloblastosis, but cases occur sporadically.

Until recently and before the recognition of subgroup J ALV (381, 394), myelocytomatosis was mainly a sporadic disease seen among young and adult birds (409). An overall incidence of 27% myelocytomatosis was reported in meat-type chickens inoculated with strain HPRS-103 of ALV-J (385). High incidence of ALV-J tumors have been reported in many countries (20, 512, 528) with mortalities up to 1.5% in excess of normal levels per week in some commercial broiler breeder flocks (206).

Of all tumors other than leukotic tumors, hemangiomas make up 25% and 19%, and nephroblastomas 19% and 3–10%, in broilers (87) and layers (409), respectively. Epizootic outbreaks of hemangiosarcomas recently have occurred in layers in Israel (79, 80).

Connective tissue tumors, which are often not the primary cause of death, make up about 20% of nonlymphoid tumors in broilers (87). The incidence of connective tissue tumors in chickens is probably less than 1 in 1000 (409), but epizootics have occurred. Outbreak of histiocytic sarcomas was reported in a flock of 600 1-year-old hens, during which tumors were found in 90% of 400 birds examined during a 4-month period (395). Low incidence of histiocytic sarcomatosis associated with ALV-J has also been reported (4).

Osteopetrosis occurs much less frequently than LL, and epizootics occur sporadically in broilers. In all types of chicken, males are more frequently affected than females. It occurs very rarely in turkeys.

Incidence of Virus Infection

Subgroup A ALV is the most common subgroup of L/S viruses isolated from field outbreaks of LL; it is encountered more frequently than subgroup B. In one study (83), 1.6–12.5% of the

embryos from 8 commercial flocks representing a variety of sources contained subgroup A ALV, and there was significant shedding in every flock. In contrast, subgroup B ALVs were relatively rare and were shed in eggs much less frequently than subgroup A. In general, fewer studies of the prevalence of ALV in meat lines have been made compared with those in egg lines. Antibodies to the novel subgroup J ALV were found in 3 of 5 meat-type chicken lines, but not in 7 layer lines examined in the United Kingdom (388). Using virological and serological assays, the incidence of ALV-J infection in affected broiler breeder flocks was reported to be as high as 87% (206). Similar high levels of ALV-J infection have been reported in other countries also (20, 310, 325, 512, 528, 570). The incidence of infection with subgroup ALV-J was also influenced by other factors such as age at exposure (560, 561).

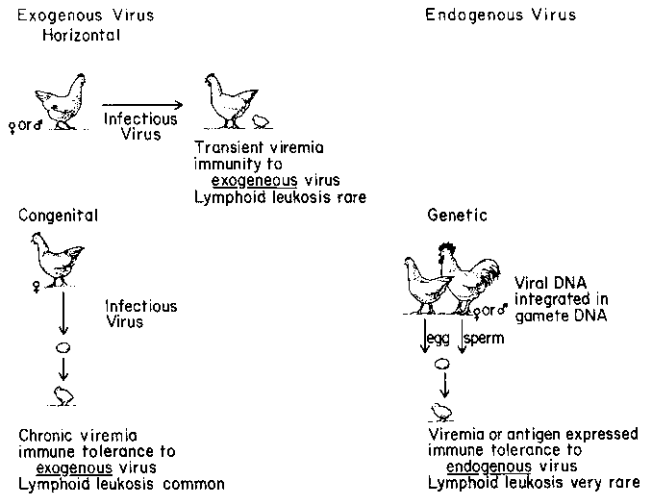
Subgroups A, B, C, and D of ALV have been isolated from commercial flocks in Finland; 5 of 10 flocks surveyed had antibody to all 4 subgroups (445).

Antibodies to subgroups A and B are common among wild-fowl and domestic chickens in Kenya and Malaysia, and some evidence exists of antibody to subgroup D viruses in Kenya. Subgroup F viruses have been found in ring-necked and green pheasants, and subgroup G viruses in Ghinghi, silver, and golden pheasants. Subgroup H virus has been isolated from Hungarian partridges and subgroup I virus from Gambel's quail (see "Virus Subgroups"). Viruses that do not fit within known subgroups have been isolated from Mongolian and Swinhoe pheasants, Chinese quail, and chickens. However, none was found in Japanese quail, pigeons, geese, and Pekin and Muscovy ducks (94).

In most vertebrate species, endogenous retroviral genomes are inherited in a Mendelian fashion and occur at distinct chromosomal loci. DNA sequences related to RAV-0, the endogenous avian retrovirus, occur in the germ lines of most domestic chickens and several species of galliform birds. For example, partridges, true pheasants, grouse, and jungle fowl contain sequences complementary to RAV-0; whereas guinea fowl, quail, peafowl, ruffed pheasants, gallo-pheasants, and turkeys do not (218). The structure, function, and regulation of endogenous retroviruses in the genome of the chicken have been reviewed (117).

Natural and Experimental Hosts

Chickens are the natural hosts for all viruses of L/S group (375); these viruses have not been isolated from other avian species except pheasants, partridges, and quail (see "Virus Subgroups"). Experimentally, however, some members of the L/S group of avian retroviruses have a wide host range and can be adapted to grow in unusual hosts by passage in very young animals or through the induction of immunologic tolerance prior to inoculation of the virus. RSV has the widest host range; it will cause tumors in chickens, pheasants, guinea fowl, ducks, pigeons, Japanese quail, turkeys, and rock partridges. Ducks were shown to be an ideal experimental system for studying persistence of ALV as the virus appeared to persist even up to 3 years with no viraemia or neutralizing antibodies after embryonic infection (350). However ducks infected as embryos with subgroup C showed wasting disease soon after hatching (496, 500, 518).



15.27. Horizontal and vertical transmission of exogenous LLV and genetic transmission of endogenous virus. (116)

There has been one report of lymphoid leukosis in ostriches (224). Some strains of RSV induce tumors in mammals (533), including monkeys (299–302). Osteopetrosis can be produced in turkeys by inoculation of fresh whole blood from affected chickens (260). Also, turkeys were susceptible to ALV-J infection and tumors induced by the acute form of strain HPRS-103 of ALV-J (531).

Transmission

Exogenous ALVs are transmitted in 2 ways: vertically from hen to progeny through the egg and horizontally from bird to bird by direct or indirect contact (113, 436, 437) (Fig. 15.27). Although usually only a small percentage of chicks are infected vertically, this route of transmission is important epizootiologically because it affords a means of maintaining the infection from one generation to the next. Most chickens become infected by close contact with congenitally infected birds. Although vertical transmission is important in the maintenance of the infection, horizontal infection may also be necessary to maintain a rate of vertical transmission sufficient to prevent the infection from dying out (382). The infection does not spread readily from infected birds to birds in indirect contact (in separate pens or cages), probably because of the relatively short life of the virus outside the birds (see "Thermal Inactivation"). However, contact exposure at hatch was shown to be an effective method of spread of ALV-J among broiler breeder chickens (206, 562, 563) and was prevented by small group rearing (566).

Four classes of ALV infection are recognized in mature chickens: (1) no viremia, no antibody (V-A-); (2) no viremia, with antibody (V-A+); (3) with viremia, with antibody (V+A+); and (4) with viremia, no antibody (V+A-) (436, 437). Birds in an infection-free flock and genetically resistant birds in a susceptible flock fall into the category V-A-. Genetically susceptible birds in an infected flock fall into one of the other three categories. Most are V-A+, and a minority, usually less than 10%, are V+A-. Most

V+A- hens transmit ALV to a varying but relatively high proportion of their progeny (386, 437). A small proportion of V+A+ hens transmit the virus congenitally and do so more intermittently; the tendency for congenital transmission of ALV in this category was found to be more frequent in hens with low antibody titer (521). Congenitally infected embryos develop immunologic tolerance to the virus and after hatching make up the V+A- class, with high levels of virus in the blood and tissues and an absence of antibodies. By 22 weeks of age, up to 25% of meat-type chickens exposed to ALV-J at hatch were found to be V+A-. Older hens (2 or 3 years of age) transmit virus in their eggs less consistently and at a lower level than birds under 18 months (78).

The role of males in the transmission of ALV is at best equivocal. Infection of the cock apparently does not influence the rate of congenital infection of progeny (436, 489). The genetics of the host and the strain of ALV influence shedding and congenital transmission after horizontal infection (133). With electron microscopy, virus budding has been seen on all structures of reproductive organs of cocks except germinal cells (150), indicating that the virus does not multiply in germ cells. The cock, therefore, acts only as a virus carrier and source of contact of venereal infection to other birds (456, 469, 488). Congenital infection of embryos is strongly associated with shedding by the hen of ALV into egg albumen and with presence of virus in the vagina of hens (386, 486). These traits are also highly correlated with viremia. Witter *et al.* (563) reported on the association between various infection profiles of subgroup J ALV in broiler breeders and transmission of virus to progeny.

Shedding of ALV into egg albumen and transmission to the embryo is a consequence of virus production by albumen-secreting glands of the oviduct. In most hens, congenitally transmitting ALV, the highest titers of virus were found in the ampulla of the oviducts, suggesting that embryo infection is closely related with ALV produced at the oviduct but not with ALV transferred from other parts of the body (520). Electron microscopy studies have revealed a high degree of virus replication in the magnum of the oviduct (155). Virus budding also occurs in various cell types in the ovary but not in the follicular cells or ovum, and transovarial infection does not seem to be important (386). Not all eggs that have ALV in the albumen give rise to infected embryos or chicks; in the studies of Spencer *et al.* (486), Payne *et al.* (386), and Tsukamoto *et al.* (521), only about one-half to one-eighth of embryos were infected from eggs with virus in the albumen. This intermittent congenital transmission may be a consequence of neutralization of virus by antibody in the yolk and of loss because of thermal inactivation. Congenital transmission of ALV has been found in the absence of detectable shedding of group-specific antigen (268).

Electron microscopy has revealed virus particles in many organs from infected chicken embryos, and virus has been observed to bud and accumulate in large amounts in pancreatic acinar cells of embryos (578). These particles, which are highly infectious, are shed in droppings of newly hatched chicks (65). Infectious virus is also present in saliva and feces of older birds that provide a source of horizontal infection to other birds (65).

In flocks infected with subgroup A ALV, only a minority of ALV-infected birds develop LL; the others remain as carriers and shedders. Viremic-tolerant (V+A-) birds are reported to be several times more likely to die of LL than those with antibody (V-A+) (436). Incidence of leukosis decreases rapidly if infection by natural routes occurs after the first few weeks of age (70); there are well-established genetic differences in susceptibility to LL development in chickens that are equally susceptible to virus infection (131).

Endogenous ALVs (see "Etiology") usually are transmitted genetically in germ cells of both sexes (Fig. 15.26). Many are genetically defective and incapable of giving rise to infectious virions, but some are not and may be expressed in an infectious form in either embryos or hatched birds. In this form, they then are transmitted similarly to exogenous viruses, although most chickens are genetically resistant to such exogenous infection. Endogenous viruses have little or no oncogenicity (346) but may influence response of the bird to infection by exogenous ALV (122, 468). Immunodepression induced by infectious bursal disease virus increased the rate of shedding of ALV (209); also strain of virus can influence incidence of tolerantly infected chickens (197).

Incubation Period

Members of the L/S group of viruses are multipotent viruses capable of inducing a variety of neoplastic diseases. The incubation period for these diseases is dependent on strain and dose of virus, route of and age at exposure and genetic constitution of the host. Susceptible chicks inoculated as embryos or at 1–14-days of age with a standard strain of ALV-RPL12 (73), B15, F42 (46), or RAV-1 developed LL between weeks 14 and 30 of age. It is very seldom that LL cases occur in chickens under 14 weeks. Certain laboratory recombinant viruses have been shown to cause LL within 5–7 weeks (285), although such short incubation periods are not found in field outbreaks. In field outbreaks, LL cases can occur any time after 14 weeks of age; however, incidence is usually highest at about sexual maturity.

Another determining factor is whether the virus strain lacks or possesses a viral oncogene. For example, diseases such as erythroblastosis, induced by slowly transforming viruses lacking oncogene, usually develop after a long latent period (96, 220, 303), as in such cases transformation is induced by promoter insertion activation of the cellular oncogene c-*erbB*. After IA inoculation of the slowly transforming RPL12 strain virus into susceptible day-old chicks, the incubation period varies from 21–110 days (73). On IV inoculation of 11-day-old embryos, chicks occasionally have been found to have erythroblastosis on hatching. Strain R virus produces a much more rapid response, and in some experiments, birds inoculated with high doses have all died between 7 and 12 days postinoculation (33). Field strains and viruses passaged in cell culture induce erythroblastosis after a longer incubation period (71). Passage from donors with erythroblastosis greatly shortens the incubation period (215).

Other strains of virus including F42 (46), ES4, and strain 13 (33) also produce erythroblastosis. Field cases usually occur in birds older than 3 months of age. Viruses such as RPL12 and F42 are nondefective and slowly transforming; whereas ES4 and R are defective and acutely transforming (243).

Strain BAI-A of ALV predominantly induces myeloblastosis. Virus stocks are defective and contain helper viruses of both A and B subgroups (272). After inoculation of susceptible day-old chicks with large doses of virus, changes in the blood can be observed in 10 days, and birds die a few days thereafter. Mortality continues for about 1 month, and only a few deaths occur after this (77, 170). The virus E26 (243) also predominantly induces myeloblastosis.

Virus-induced myelocytomatosis generally has a longer incubation period than erythroblastosis and myeloblastosis induced by the acutely transforming virus strains, but shorter than LL. On IV injection of MC29 into young chicks, myelocytomas were obtained in 3–11 weeks (338). The incubation period in field cases is unknown, but most cases are observed in immature birds. The virus CMII also induces myelocytomas (243). Myelocytomatosis induced by the HPRS-103 strain of ALV, which lacks a viral oncogene, had a long latent period (median time to death was 20 weeks) (383). However, median time to death with the acutely transforming 879-strain variant of HPRS-103, believed to carry a viral oncogene, was 9 weeks (384). Field cases of subgroup J-induced myelocytomatosis were reported in broiler breeder chickens as young as 4 weeks of age (206).

Most strains of ALV have been found to cause hemangiomas (66, 216). These tumors can be found in birds of various ages. In naturally occurring outbreaks, most mortality from hemangiosarcomas occurred at 6–9 months (79, 80). Induction of lung angiosarcomas by subgroup F ALVs is reported (465). After experimental inoculation of young chicks with field strains of virus (215), hemangiomas appeared in 3 weeks to 4 months.

In field cases, ALV-induced renal tumors are rarely seen in chickens younger than 5 weeks; most cases are seen in birds between 2 and 6 months of age. Nephroblastomas induced by strain BAI-A may reach an incidence of 60–85% in birds not dying of myeloblastosis (77). Renal carcinomatous lesions induced by strain MC29 are found as soon as 18 days or as late as 7 weeks after virus inoculation. Incidence in inoculated chickens may be 60% or more, but incidence in field flocks is not known.

Osteopetrosis may develop any time after 1 month following experimental inoculation of day-old chicks with strain RPL12-L29 of ALV (447) or other viruses (258, 260, 406); it is most commonly seen in birds 8–12 weeks of age. The disease probably has a similar incubation period in the field. MAV-2(O) virus will induce palpable osteopetrosis 7–10 days after hatching in chicks inoculated at 1 day of age or as 11–12-day-old embryos (214).

Sarcomas may occur any time after inoculation with ALVs but are most frequently observed in the first 2–3 months (76). In field flocks, connective tissue tumors may occur in birds at any age (576). Sarcomas also develop readily and are palpable within 3 days after inoculation of chicks with high doses of acutely transforming RSV.

Clinical Signs

Outward signs of the leukotic diseases are mostly nonspecific. They include inappetance, weakness, diarrhea, dehydration, and emaciation. In LL especially, there may be abdominal enlargement. The comb may be pale, shriveled, or occasionally cyanotic. In erythroblastosis and myeloblastosis, hemorrhage from feather follicles also may occur. After clinical signs develop, the course is usually rapid, and birds die within a few weeks. Other affected birds may die without showing obvious signs.

In myelocytomatosis, skeletal myelocytomas may cause protuberances on the head, thorax, and shanks. Myelocytomas may occur in the orbit of the eye, causing hemorrhage and blindness. Hemangiomas may occur in the skin, appearing as "blood blisters," and these may rupture causing hemorrhage. Renal tumors may cause paralysis due to pressure on the sciatic nerve. Sarcomas and other connective tissue tumors may be seen in the skin and musculature. When advanced, these various other tumors may be accompanied by the nonspecific signs given previously. Benign tumors may follow a long course, malignant tumors a rapid one.

In osteopetrosis, the long bones of the limbs are commonly affected (Fig. 15.45). Uniform or irregular thickening of the diaphyseal or metaphyseal regions can be detected by inspection or palpation. The affected areas are often unusually warm. Birds with advanced lesions have characteristic "bootlike" shanks. Affected birds usually are stunted and pale and walk with a stilted gait or limp.

In recent years, ALV has been shown to be associated with the "so called fowl glioma" (359), associated with cerebellar hypoplasia and myocarditis (253, 273, 274, 514–517).

Pathology

Introduction

One or more specific neoplasms induced by ALSVs may occur in a given flock of chickens, and more than one type of neoplasm may occur in an individual bird. This is particularly true in flocks infected with subgroup J ALV. The presence of a tumor similar to that produced experimentally is only provisional evidence that a bird was infected with a virus of this group. Firmer evidence is provided by ALSV detection or isolation, and if appropriate by experimental reproduction of tumors by a virus isolate.

In this section, the pathology of the different neoplasms is discussed without regard for virological properties of the inducing agent(s). Only entities that have been reproduced with ALSVs are described.

Nonneoplastic Conditions

The clinical consequences of infection of chickens with exogenous ALV vary. Some chickens, principally those with a tolerant viremic infection (arising from congenital or early neonatal infection, see "Immunity"), may show a variety of clinical signs, as detailed later in this chapter, including depression of body weight and of other production traits. Birds with tolerant viremic infections are also those most likely to develop neoplasia. Ultrastructural and virological studies of congenitally or neonatally infected birds have shown the virus to be widespread in most tissues and organs of the body (5, 6, 156, 165, 426, 495, 516). Dougherty and DiStefano (155–157, 165) observed virus budding in cells of every type of tissue examined, except germ cells and neurons. In contrast, other chickens, particularly those that

develop an immune response, show no obvious clinical signs of infection, even though the virus persists in the body, although in lower amounts and at fewer sites (5).

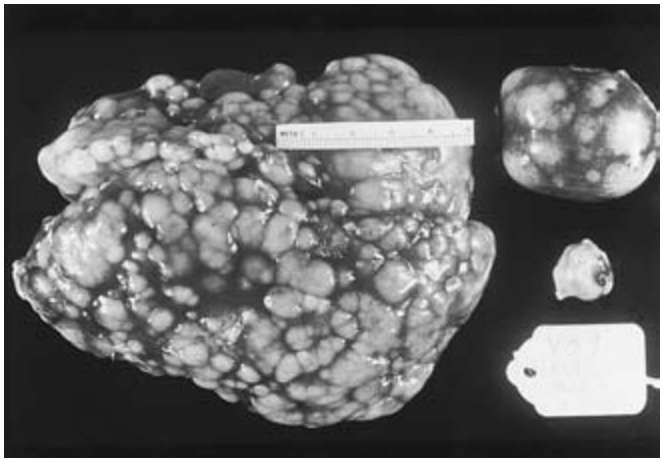
ALV infection in the absence of overt disease can adversely affect the productivity of egg-laying chickens. Compared with nonshedders, hens that shed virus produced 20–35 fewer eggs per hen housed to 497 days of age; matured later sexually (i.e., age at first egg); and produced smaller eggs (485), at a lower rate, and with thinner shells. Mortality from causes other than neoplasms was 5–15% higher, fertility was 2.4% lower, and hatchability was 12.4% lower in shedders than nonshedders (228). In this study, shedding refers to the transfer of ALV to egg albumen and nonshedders, as well as shedders, and would likely be ALV-infected; shedders are mostly viremic birds; whereas nonshedders are immune with ALV antibody. ALV infection has similar effects on broiler breeders and causes a consistent, although often small, reduction in broiler growth rate (130, 227). However, these effects are more marked in meat-type chickens infected with subgroup J ALV (491, 492, 494, 495). In broiler breeder flocks affected by myeloid leukosis caused by subgroup J ALV, smaller eggs were associated with the presence of gs antigen in allantoic fluid and virus in the embryo (485). Other studies on reduced productivity in chickens with ALV infections and the genetic consequences have been reviewed (226, 270, 484). The presence of ALV in semen was not associated with reduced semen production, but some evidence suggested an effect on semen quality and fertility (456). The physiological bases for these effects have not been studied.

A number of other nonneoplastic effects of ALV infection have been observed, mostly in experimental infections.

Chickens, turkeys, and jungle fowl exposed when young to certain ALVs (RAV-1, RAV-60, MAV-2(O), and ALVs of subgroup B and D develop anemia, hepatitis, immunodepression, and wasting; some may die (122, 479). A myocarditis and chronic circulatory syndrome was reported in chickens inoculated with RAV-1 ALV (232). Intracytoplasmic viral matrix inclusion bodies have been observed in the myocardium of adult ALV infected chickens (235, 348). Chickens inoculated with RAV-7 develop neurologic signs including ataxia, lethargy, and imbalance resulting from a nonsuppurative meningoencephalomyelitis (557). A persistent infection of the central nervous system, with inflammatory lesions and clinical signs, followed *in ovo* infection with RAV-1 (186). Fowl glioma-associated virus also shows involvement of brain with cerebellar hypoplasia (516).

In MAV-2(O) infection, anemia occurs due to an aplastic crisis in the bone marrow in which erythrocytes fail to incorporate iron into hemoglobin and exhibit a decreased survival time (140). Administration of antiviral antibody will prevent anemia (406). The immunodepression may involve atrophy or aplasia of lymphoid organs, hypergammaglobulinemia, decreased mitogen-induced blastogenesis, and decreased antibody response (479). The changes in the immune system are likely a result of cessation of B-cell maturation and a block in the development of T-suppressor cells, possibly due to interference with the synthesis of functional interleukin-2 (258, 307).

In addition to stunting and atrophy of the lymphoid organs, RAV-7 caused obesity, high triglyceride and cholesterol levels, reduced thyroxine levels (hypothyroidism), and increased insulin levels. The frequent occurrence of stunting may relate to the virus's suppression of thyroid function. Stunting of chicks with congenital subgroup J ALV infection was also associated with hypothyroidism, possibly mediated via effects on the pituitary (59) or other effects (491, 492, 494).



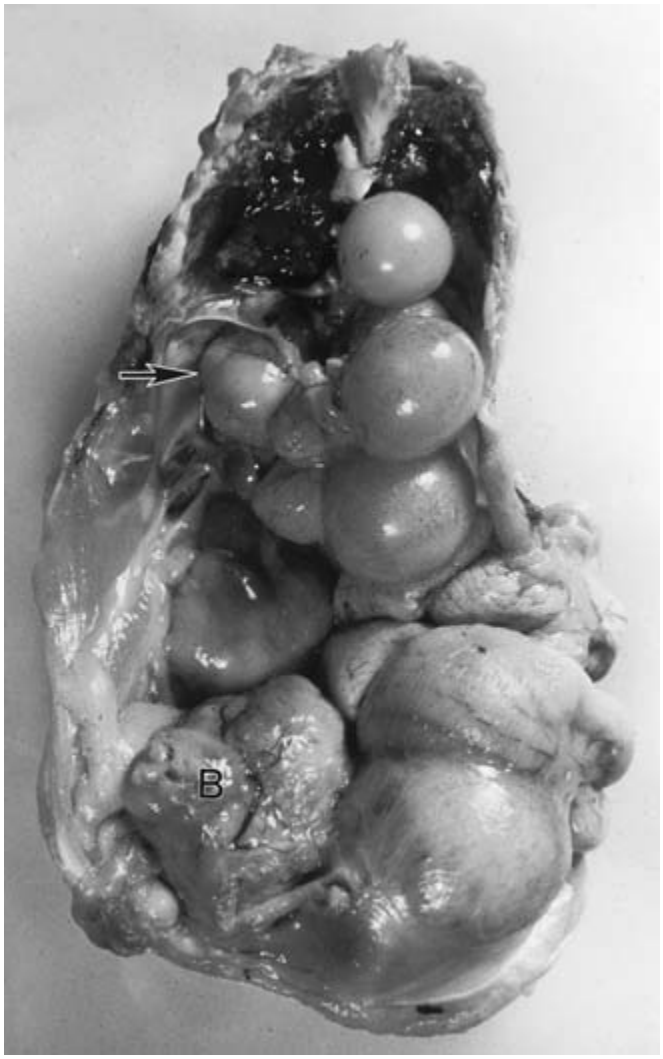
15.28. Nodular lesions in liver and spleen of bird with LL inoculated at 1 day of age with RPL12 virus. Bursa also has a small tumor.

Lymphoid Leukosis

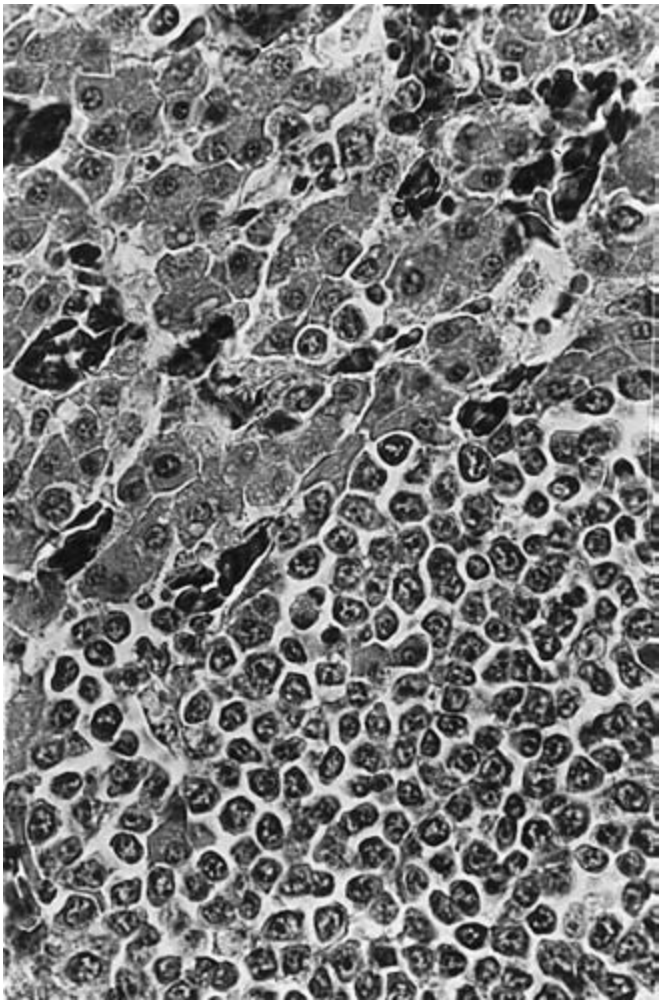
Gross. Fully developed LL occurs in chickens of about 4 months of age and older. Grossly visible tumors almost invariably involve the liver (Figs. 15.28 and 15.33A), spleen, and bursa of Fabricius (Figs 15.29 and 15.33G). Other organs often grossly involved include kidney, lung, gonad, heart, bone marrow, and mesentery.

Tumors are soft, smooth, and glistening; a cut surface appears grayish to creamy white and seldom has areas of necrosis. Tumor growth may be nodular (Fig. 15.28), miliary, diffuse (Fig. 15.33A), or a combination of these forms. In the nodular form, the lymphoid tumors vary from 0.5–5 cm in diameter and may occur singly or in large numbers. They are usually spherical but may be flattened when they are close to the surface of an organ. The miliary form, which is most obvious in the liver, consists of numerous small nodules less than 2 mm in diameter uniformly distributed throughout the parenchyma. In the diffuse form, the organ is uniformly enlarged, slightly grayish in color, and usually very friable. Occasionally, the liver is firm, fibrous, and almost gritty.

Microscopic. All tumors are focal and multicentric in origin. Even in organs appearing diffusely involved when examined grossly, the microscopic pattern is one of coalescing foci. As tumor cells proliferate, they displace and compress cells of the organ rather than infiltrate between them (Fig. 15.30). Nodules in the liver usually are surrounded by a band of fibroblast-like cells that have been shown to be remnants of sinusoidal endothelial cells (245). In the bursa, a follicular pattern of tumor growth usually can be seen.



15.29. Large tumor of bursa of Fabricius (B) and kidneys (arrow) in a naturally occurring case of lymphoid leukosis in the adult hen.



15.30. Liver tumor from a 20-week-old chicken inoculated at 1 day of age with RAV-1 ALV. Note displacement and compression of hepatic parenchyma. ×700. (191)

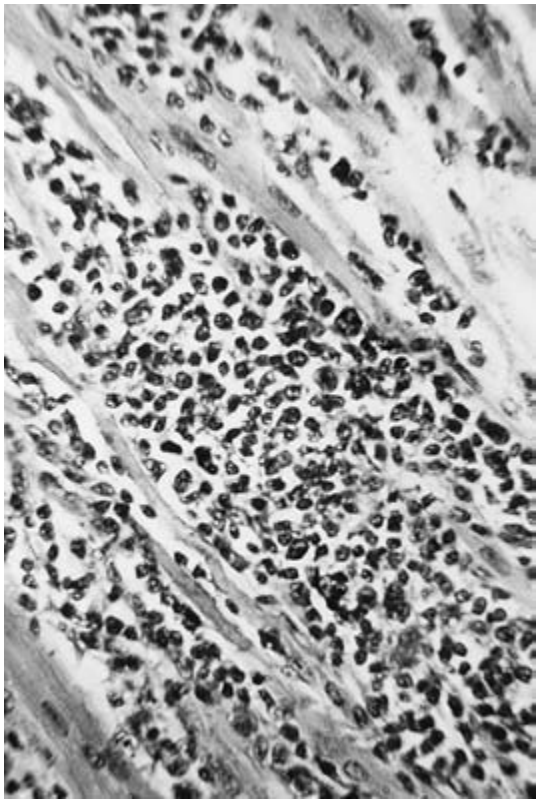
Tumors consist of aggregates of large lymphoid cells (lymphoblasts) that may vary slightly in size but are all at the same early developmental stage. They have a poorly defined cytoplasmic membrane, much basophilic cytoplasm, and a vesicular nucleus in which there are margination and clumping of the chromatin and one or more conspicuous acidophilic nucleoli (374).

The cytoplasm of most tumor cells contains a large amount of RNA, which stains red with methyl green pyronin, indicating that the cells are immature and rapidly dividing (109–111). Characteristic features of the cell can best be seen in wet-fixed impression smears that have been stained with May-Grunwald-Giemsa, methyl green pyronin, or other cytological stains. The tumor cells have B-cell antigen markers and produce and carry IgM on their surface (113, 393).

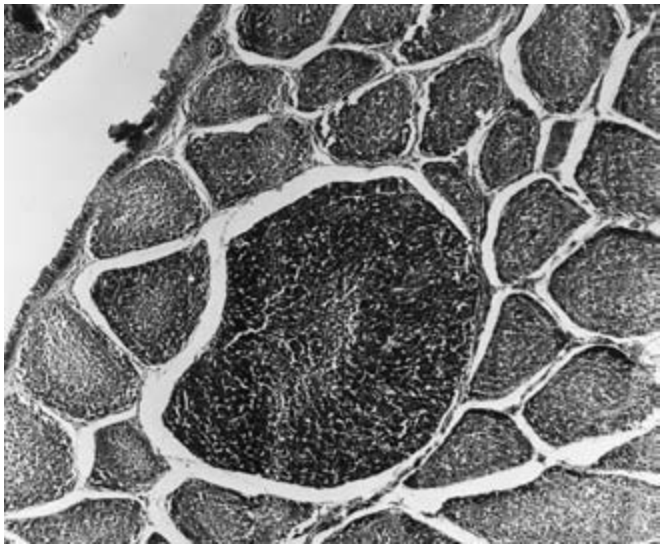
No consistent or significant changes occur in cellular elements of circulating blood. Rarely, lymphoblasts predominate in frank leukemic cases.

Ultrastructural. Vacuoles are found infrequently in lymphoid cells of birds with LL, but some virus particles have been observed budding from the plasma membranes of lymphoblasts (158–160, 165).

Pathogenesis. ALVs multiply in most tissues and organs of the body (165). Transitory lymphoid foci may occur in various tissues and are considered to be inflammatory in nature (82) (Fig. 15.31). The infection persists longer in bursal lymphocytes than in other hematopoietic tissues (10, 11), and cells of the bursa of Fabricius are the target cells that neoplastically transform. The target cells must be resident in the bursa, because surgical bursectomy up to 5 months of age and other treatments that destroy the bursa of Fabricius will eliminate the disease (64, 98, 284, 396, 410, 412, 429). Medullary macrophages appear to be the principal bursal cells for virus replication and may be important in transmitting infection to the lymphoid cells (234). At a variable time after infection, which can be as short as 4 weeks in ex-



15.31. Lesions in young chickens induced by leukosis virus. Heart from a 4-week-old RIF3-infected chick. Diffuse accumulations of lymphoid cells among myocardial fibers. ×430. (Calnek)



15.32. Lymphoblastic transformation in single bursal follicle in chicken with lymphoma leukosis (LL). All surrounding follicles are histologically normal in this and other sections from a 16-day-old chicken infected with RPL12 virus at hatching. Methyl green pyronin, ×40. (Dent)

perimental studies, a proliferation of lymphoblasts occurs in one or more lymphoid follicles in the bursa. These altered bursal follicles are termed transformed follicles (10, 352, 409), and the change is regarded as a focal preneoplastic hyperplasia (262, 263) (Fig. 15.32). The transformed follicle is a consequence of activation of the c-*myc* gene by nearby insertion of ALV. This places the c-*myc* gene under the control of the enhancers of the viral LTR, resulting in overexpression of *myc*, causing a maturation arrest and proliferation of bursal stem cells, associated with changes in global gene expression profiles and genomic instability (353, 354). Arrest of maturation of the transformed B cells results in interference of the normal intraclonal switch of immunoglobulin production from IgM to IgG, hence the surface IgM that characterizes LL cells. The cells grow within the confines of the bursal follicle and are not neoplastic. Sometimes, many follicles are transformed, but the majority of these appear to regress, and only a few continue to grow to give rise to nodular tumors in the bursa, which are visible grossly from about 14 weeks of age (112, 352). Progression of the transformed follicle to the fully neoplastic state requires additional genetic changes, and other putative oncogenes, *Blym*-1 (239, 351) Mtd/Bok (57) and c-*bic* (104, 502), have been implicated. Recent studies have suggested that the oncogenicity associated with the non-coding c-*bic* transcript is due to a novel microRNA designated miR-155 (503). Oncogenicity assays demonstrated that *bic* can cooperate with c-*myc* in lymphomagenesis and erythroleukemogenesis, providing direct evidence for the involvement of untranslated RNAs in oncogenesis (504). Evidence suggests that apoptosis of neoplastic bursal cells is inhibited by an antagonist of apoptotic cell death, NR-13, related to the *Bcl*-2 proto-oncogene (316). Induction of angiogenic factors from the transformed cells also contributes to the generation of *myc*-induced lymphomas (55). From about 12 weeks of age, cells in the clonal bursal tumors metastasize to other organs and tissues and result in the terminal disease. Metastatic tumors in the viscera usually have the same DNA fragments as bursal tumors from the same birds, supporting their clonal origin (124), but multiple bursal tumors can give rise to polyclonal metastatic disease (474).

Experimentally, B-cell lymphomas also have been induced by c-*myb* activation, following embryonic infection with ALV (285, 401). The tumors were unusual in that metastatic disease occurred within 7 weeks of infection, and preneoplastic and primary bursal neoplasms were not detected. Spontaneous bursal lymphomas of unknown etiology have also been observed in lines of chickens free of exogenous ALV including line 0 free of *ev* loci (138, 139). REV (see "Reticuloendotheliosis") can also induce LL associated with c-*myc* activation.

Transplantable LL tumors can be developed from ALV-induced tumors. The RPL12 transplantable tumor, from which the RPL12 strain of ALV was isolated, is a well-known example. Several other new LL transplantable tumors have been described (365). Transplantable LL tumors grow to a palpable size within 5–10 days and become widely disseminated, inducing rapid mortality.

Erythroblastosis

Gross. Natural cases of erythroblastosis (erythroid leukosis) usually occur in birds between 3 and 6 months of age. The liver and kidney are moderately swollen, and the spleen often is greatly enlarged. The enlarged organs are usually cherry red to dark mahogany (Fig. 15.33) and are soft and friable. The marrow is hyperplastic, semi-liquid, and red in color. Petechial hemorrhages occur in various organs such as muscles, subcutis, and viscera. Thrombosis, infarction, and rupture of the liver or spleen may be observed. Edema of the lungs, hydropericardium, and a fibrinous clot on the liver may occur.

With severe anemia, atrophy usually is seen in visceral and lymphoid organs, particularly the spleen.

Changes in the blood reflect those in other organs, such as liver, spleen and bone marrow, and depend largely on the extent of anemia or leukemia. When severe anemia exists, the blood is watery and light red and clots slowly. In contrast, acute cases may show no grossly apparent changes, although usually the blood appears dark red with a smoky overcast.

Microscopic. Examination of the marrow in early cases reveals blood sinusoids filled with rapidly proliferating erythroblasts that fail to mature. In advanced cases, marrow consists of sheets of homogeneous erythroblasts with small islands of myelopoietic activity and little or no adipose tissue. With concurrent anemia, the number of erythropoietic cells may be reduced.

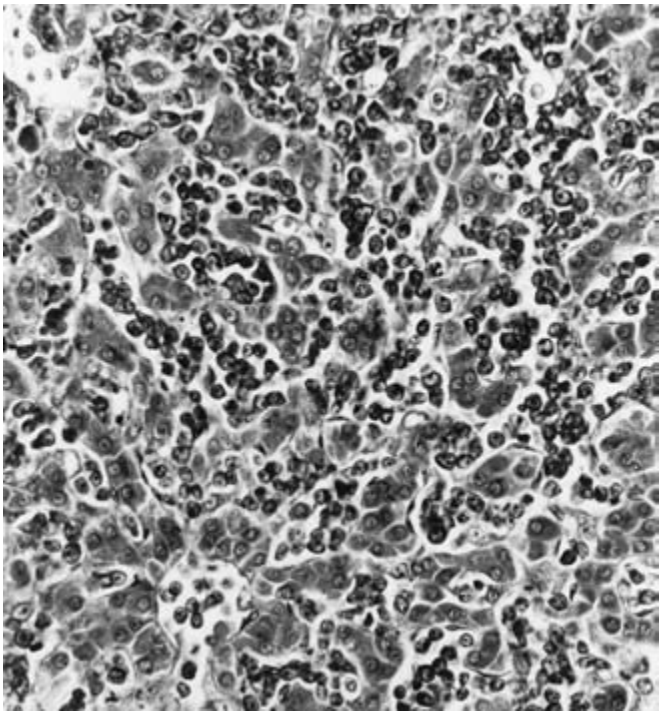
Alterations in visceral organs are primarily due to hemostasis, resulting in an accumulation of erythroblasts in the blood sinusoids and capillaries (Fig. 15.34). The liver sinusoids, splenic red pulp, bone marrow, and sinusoids of other organs are filled with proliferating erythroblasts.

The sinusoids become greatly distended, resulting in pressure atrophy of the parenchyma. Although accumulations of erythroblasts may be extensive, they always remain intravascular, unlike those in LL and myeloblastosis.

Varying degrees of anemia may occur. Sometimes erythroblastosis occurs, and there may be only severe anemia. Extramedullary erythropoiesis is common.

The primary cell involved is the erythroblast. The cell has a large round nucleus with very fine chromatin, 1 or 2 nucleoli, and a large amount of cytoplasm that is basophilic. A perinuclear halo, vacuoles, and occasionally fine granules are present. The cell is irregular in shape and often has pseudopodia. Erythroblasts have cell markers that identify them as members of the erythrocytic series.

Stained blood smears reveal a variable number of erythroblasts (see Fig. 15.33D). These vary in maturity from the early erythroblast, which is the dominant cell, to the various stages of polychrome erythrocytes. The more mature cells often appear early in the course of the disease or during remission, if it occurs. The thrombocytic series of cells may be somewhat increased in number and immaturity. Similarly, in most naturally occurring cases, immature cells of the myelocytic series appear in the peripheral circulation. Occasionally, they are as prominent as the erythroblasts. Cases of mixed erythroblastosis and myelocytomatosis may occur.

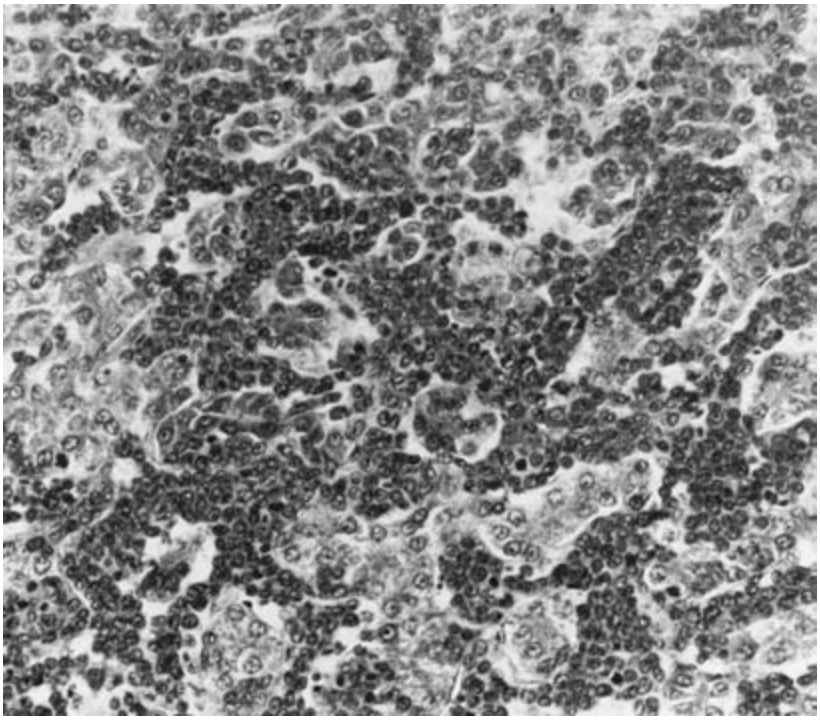


15.34. Erythroblastosis. Liver sinuses permeated with erythroblasts in bird 40 days after inoculation with strain MC29 leukosis virus. ×280. (Beard)

Ultrastructural. Numerous studies have been made of the primitive cells in erythroblastosis induced by different strains of ALV (35, 36, 249) and RPL12 (160). Neoplastic erythroblasts are for the most part indistinguishable from corresponding cells in the normal bird, except that virus particles may be present in extracellular spaces and within vacuoles inside cells. In erythroblasts in the circulating blood, as in cell culture, there is a great increase in membrane activity, with vacuolization of the cytoplasm and budding of virus particles from the cell membrane. Only occasionally are aberrant structures seen in erythroblasts (160).

Pathogenesis. Inoculation of slowly transforming strains (i.e., those lacking a viral oncogene) of ALV such as RPL12 into 11-day-old chick embryos induces erythroblastosis from the first week of age (400). When day-old chicks are inoculated, the incubation period varies from 21 to more than 100 days (73). Induction of erythroblastosis by slowly transforming ALV involves activation of the cellular oncogene c-*erb*B by LTR insertion (96, 221, 306), and new acutely transforming AEV strains with transduced c-*erb*B genes may arise (257, 334). Whether such acutely transforming viruses spread naturally and induce more erythroblastosis is not clear.

Experimentally, acutely transforming AEV strains, such as ES4 and R, cause mortality from erythroblastosis 7–14 days after inoculation (243). ES4 carries the gene v-*erb*A, which blocks erythroid precursor cell differentiation, in addition to the v-*erb*B oncogene (142, 181, 319, 331). Two subgroup J ALV isolates, 1B and 4B, have been shown to be acutely transforming and to in-



15.35. Myeloblastosis. Distribution of myeloblasts in liver of bird with myeloblastic leukemia 19 days after inoculation with BAI-A virus. ×280. (Langlois)

duce erythroblastosis as well as myelocytomatosis and other tumors (530). Their viral oncogenes have not yet been identified.

When birds are exposed to an acutely transforming AEV, the first alterations are found in 3 days as foci of proliferating erythroblasts in bone marrow sinusoids. By day 7, the primitive cells reach the circulating blood, and some foci of erythroid proliferation are present in sinusoids of the liver and spleen. Erythroblasts continue to accumulate in hepatic sinusoids and elsewhere until death of the host and transplantable erythroblastosis tumors can be developed (402).

Myeloblastosis

Gross. Natural cases of myeloblastosis (myeloblastic myeloid leukosis) are uncommon and usually occur in adult chickens. The liver is greatly enlarged and firm with diffuse grayish tumor infiltrates, which give a mottled or granular ("Morocco leather") appearance (Fig. 15.33C). The spleen and kidneys are also diffusely infiltrated and moderately enlarged. The bone marrow is replaced by a solid, yellowish-gray tumor cell infiltration.

A severe leukemia exists, with myeloblasts comprising up to 75% of peripheral blood cells and forming a thick buffy coat and usually an anemia and thrombocytopenia.

Microscopic. Parenchymatous organs, notably the liver, show a massive intravascular and extravascular accumulation of myeloblasts with a variable proportion of promyelocytes (Fig. 15.35). In the spleen, these tumor cells accumulate in the red pulp. In the bone marrow, myeloblastic activity is confined to extrasinusoidal areas.

Myeloblasts in leukemic blood smears are large cells with slightly basophilic clear cytoplasm and a large nucleus containing 1–4 acidophilic nucleoli, which do not stain prominently (Fig. 15.33E). Often, promyelocytes and myelocytes are also present; they easily can be identified by their specific granulation, which in the early forms is primarily basophilic. The disease may result in a secondary anemia, with the presence of polychrome erythrocytes and reticulocytes. Such a secondary anemia is distinguished easily from the conditions in which erythroblastosis and myeloblastosis occur together, because then blast cells of both cell series are present in the circulating blood.

Ultrastructural. In circulating myeloblasts from birds with myeloblastosis induced by BAI-A AMV, virus particles are only rarely found and then in small numbers in clear vacuoles (33, 160, 249, 250). However, reticular and phagocytic elements of the spleen and bone marrow frequently are packed with virus particles. When myeloblasts are transferred to cell culture, large numbers of lysosomes appear in the cytoplasm. After some time in cell culture, virus particles can be seen in lysosomes, in vacuoles, and budding at the cell membrane. No other changes are observed in these cells.

Pathogenesis. The v-*myb* gene of AMV is responsible for neoplastic transformation of the target myeloblasts (181). Experimental infection is followed within a few days by the appearance of multiple foci of proliferating myeloblasts in the extrasinusoidal areas of the bone marrow, followed rapidly by leukemia and infiltration of the liver, spleen, and other organs (308, 309).

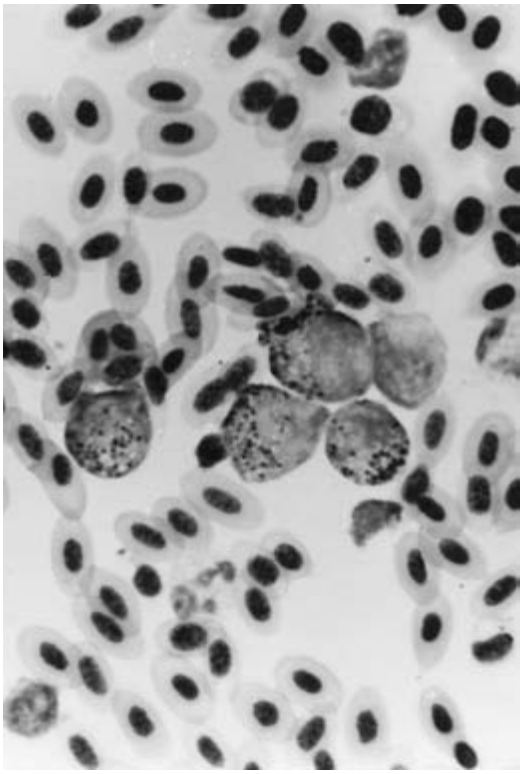
Myelocytomatosis

Gross. Tumors of myelocytomatosis (myelocytic myeloid leukosis) are distinctive and can be recognized on gross examination with some degree of certainty. Characteristically, they occur on the surface of bones in association with the periosteum and near

cartilage, although any tissue or organ can be affected. Myelocytomas often develop at the costochondral junctions of the ribs, on the inner sternum, pelvis, and on the cartilaginous bones of the mandible and nares. Flat bones of the skull are also commonly affected (Fig. 15.33H). Tumors may also be seen in the oral cavity, trachea, and in and around the eye (403). The tumors are usually nodular and multiple, with a soft, friable consistency and of creamy color. In the disease caused by subgroup J ALV, myelocytomatous infiltration often causes enlargement of the liver and spleen and other organs, in addition to skeletal tumors (559). Myelocytic leukemia may also occur (381).

Microscopic. Tumors consist of masses of uniform, usually well-differentiated, myelocytes. Their nuclei are large, vesicular, and usually eccentrically located, and a distinct nucleolus is usually present. The cytoplasm is usually tightly packed with acidophilic granules, which are usually spherical. When imprint preparations of fresh tumors are stained with May-Grunwald Giemsa, granules appear brilliant red (Fig.15.33F). Areas of less well-differentiated myelocytes are not uncommon within the myelocytomas, and areas of undifferentiated cells, which may be stem cells of the myelocyte-monocyte series, may also be found. In the liver, accumulations of neoplastic myelocytes occur around blood vessels and in the parenchyma. In the spleen, tumor cells are present in the red pulp. In the marrow, the extrasinusoidal myelopoietic areas are greatly expanded by uniform neoplastic myelocytes. A detailed description of bone and bone marrow lesions in myelocytomatosis apparently caused by subgroup J ALV is provided by Nakamura *et al.* (349).

Although the naturally occurring disease has been stated to be usually aleukemic, myelocytomatosis induced by subgroup J ALV frequently is accompanied by a marked leukemia of myeloid cells. Laboratory strains of myelocytomatosis-inducing virus, such as MC29, also cause leukemia (Fig. 15.36).



15.36. Myelocytomatosis. Granulated myelocytes in blood smear from bird 23 days after inoculation with strain MC29 leukosis virus. ×750. (Beard)

Ultrastructure. Ultrastructural features of myelocytoma cells vary from those of well-differentiated myelocytes to those of undifferentiated, nongranulated myeloid cells (338).

Pathogenesis. Acutely transforming strains of ALV that induce myelocytomatosis, such as MC29 and CMII, carry the v-*myc* oncogene (181, 342). Slowly transforming strains of subgroup J ALV that also induce myelocytomatosis, such as HPRS-103 and ADOL-Hc1, do not carry an oncogene, but molecular studies of HPRS-103-induced myelocytomatosis indicate that c-*myc* is activated (95–97). The acutely transforming strain 966 ALV, derived from myelocytoma and induced by strain HPRS-103 of subgroup J ALV, has been shown to carry v-*myc* (95, 384). Studies on HPRS-103 and 966 showed that they have a tropism for the myelomonocytic cell lineage, which may relate to their ability to cause myelocytomas (5, 6). Recently, a recombinant ALV with envelope of subgroup B and LTR of subgroup J (ALV-B/J) was isolated from a field outbreak of myeloid leukosis in commercial layers (236). However, inoculation of experimental and commercial strains of white leghorn chickens with this recombinant ALV-B/J resulted in primarily LL, but not myeloid leukosis, suggesting that differences in the genetic makeup of the commercial layers from which ALV-B/J was originally isolated and lines of chickens used in experimental inoculations studies may be responsible for the differences in pathogenicity observed (321).

The earliest alterations occur in bone marrow in which there is crowding of intrasinusoidal spaces, principally by myelocytes, and destruction of sinusoid walls. The spaces may contain 2 types of cells—the primitive hemocytoblast-like cell (myeloid stem cell) and the neoplastic myelocyte. The latter appears to arise directly from the stem cell, and differentiation is arrested both at the nongranulated and granulated myelocyte level (338). Myelocytes proliferate and soon overgrow the bone marrow. Tumors form by expansion of marrow growth and may crowd through the bone and periosteum. Extramedullary tumors may also arise by blood-borne metastasis.

Hemangioma

Gross. This tumor is found in the skin or in visceral organs in chickens of various ages. They appear as blood-filled cystic masses (blood blisters) (Fig. 15.37) or more solid tumors and consist of distended blood-filled spaces lined by endothelium or as more cellular, proliferative lesions (86). They are often multiple and may rupture, causing fatal hemorrhage (480).

Microscopic. The cavernous form is characterized by greatly distended blood spaces with thin walls composed of endothelial



15.37. Hemangioma of gizzard serosa of RPL12 virus-inoculated bird. Note the dark circumscribed and raised tumor nodules. (245)



15.38. Cavernous hemangioendothelioma of mesentery. (Feldman and Olson)

cells (Fig. 15.38). Capillary hemangiomas are solid masses in which endothelium may proliferate into dense masses (hemangioendothelioma), leaving mere clefts for blood channels (Fig. 15.39); develop into a lattice with capillary spaces; or grow into collagen-supported cords with larger interspersed blood spaces. Solid and papillary forms have also been described (347).

Ultrastructural. Hemangiomas consisted mainly of undifferentiated mesenchymal cells and had an alveolar structure (327).

Pathogenesis. Sequence analysis of an avian hemangioma-inducing virus isolated from layer hens revealed unique elements in both *env* gene and LTR that were thought probably responsible for its biologic and pathogenic characteristics (80). This virus was cytocidal and had an affinity for endothelial cells (418, 419). Apart from this, the molecular basis for hemangioma induction is not known.

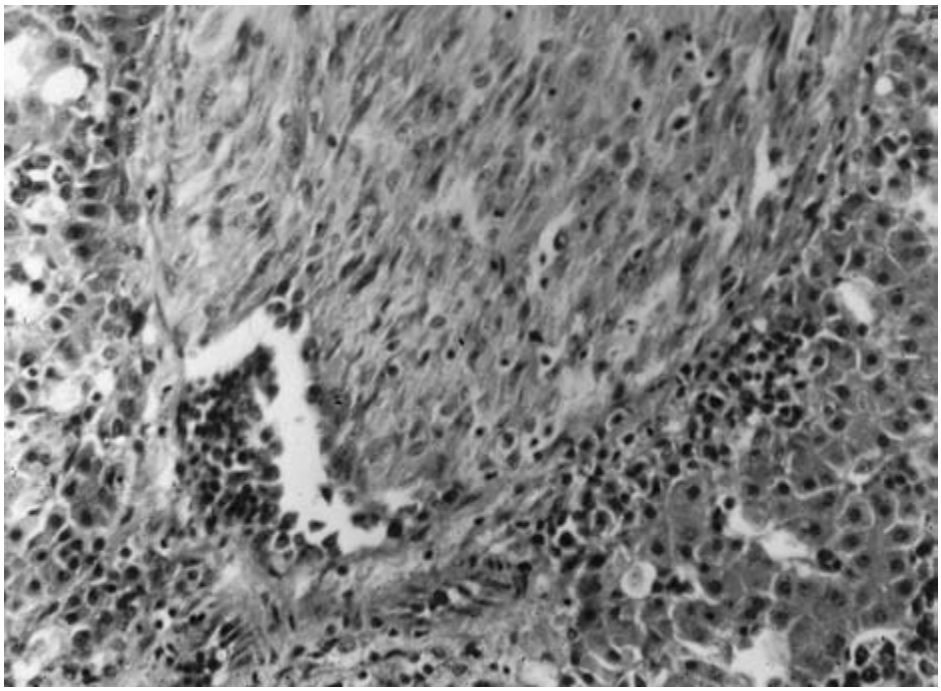
Hemangiomas are tumors of the vascular system and as such usually involve all layers of blood vessels. In some instances, the endothelium may proliferate more than the supporting tissue. Hemangiosarcomas have been associated with subgroup J ALV infection (237, 377).

Nephroma and Nephroblastoma

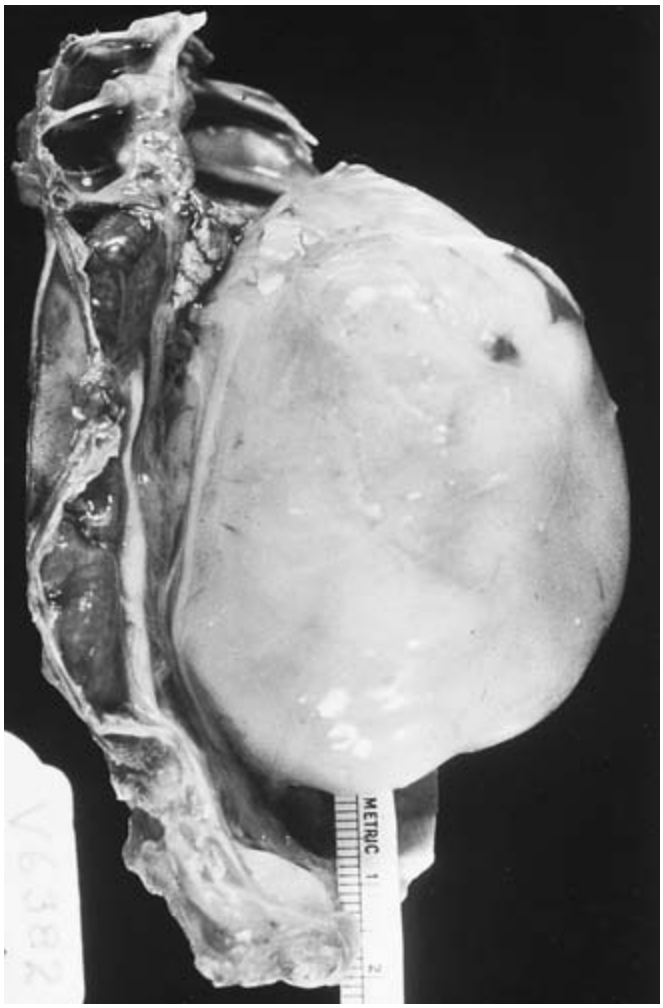
Gross. Two types of renal tumor occur: nephroblastomas (Wilms' tumor) and adenomas and carcinomas. Nephroblastomas vary from small, pinkish gray nodules embedded in the kidney parenchyma to large, yellowish gray lobulated masses that replace most of the kidney tissue (Fig. 15.40). Tumors may be pedunculated and connected to the kidney by a thin fibrous vascular stalk. Large tumors are often cystic and may involve both kidneys. Adenomas and carcinomas vary in size and appearance, similar to nephroblastomas. They are often multiple and within cysts.

Microscopic. In nephroblastomas, the histologic variation between different tumors or areas of the same tumor is striking. There is usually neoplastic proliferation of both epithelial and mesenchymal elements, although their proportion and differentiation vary widely. Epithelial structures vary from enlarged tubules with invaginated epithelium and malformed glomeruli; through irregular masses of distorted tubules; to groups of large, irregular, cuboidal, undifferentiated cells with little tubular organization (Fig. 15.41). The growth may be embedded in a loose mesenchymal or sarcomatous stroma. There may be islands of keratinizing stratified squamous epithelial structures (epithelial pearls), cartilage, or bone (148, 256, 271). Primary multiplicity of tumors may occur, but metastases are rare.

Adenomatous or carcinomatous growths also vary greatly in microscopic appearance. In tubular adenocarcinomas, primitive



15.39. Endothelioma in liver of bird inoculated with RPL30 leukosis virus. Occlusion of portal vein by inward-growing spindle cells from blood vessel. ×250. (Fredrickson)



15.40. Nephroblastoma. Bird was inoculated at 1 day of age with avian myeloblastosis virus (AMV) (BAI-A).



15.41. Nephroblastoma. Bird was inoculated at 1 day of age with cloned preparation of avian myeloblastosis virus (AMV) (BAI-A). Note primary multiplicity of tumors of two distinct types in different areas (arrows). ×20

abnormal glomeruli frequently occur in large numbers among abnormal tubules. Papillary cyst adenocarcinomas are frequent. At times, solid carcinomas with little evidence of renal tubules develop (37, 338). Rarely is there cartilage and never other mesenchymal tumor tissues. A trabecular fibrous tissue stroma may separate masses of epithelial tumor tissue.

Ultrastructural. In the epithelial nephronic elements of nephroblastomas induced by strain BAI-A of ALV (33), cytoplasmic aberrant structures occasionally are seen in large or small aggregates. Virus particles bud from cell membranes of epithelial cells, fibroblastic elements of the stroma, and chondrocytes. Sarcomatous elements consist of cells similar in morphology to those in other avian sarcomas. Virus particles have been observed budding from epithelial cells in cystadenomas and adenocarcinomas induced by strain MC29 myelocytomatosis virus (340). Large accumulations of particles in spaces in the cysts and tubules probably were related to a lack of tubule and glomerular drainage.

Pathogenesis. A target oncogene for ALV-induced nephroblastomas was not consistently identified (106). More recently, a new proto-oncogenes such as *nov*, (281) and *twist* (366) were identified as common integration sites in nephroblastomas (367).

Nephroblastomas originate from nephrogenic blastema (embryonic nephrons and embryonal rests) (86, 271). This blastema tissue is present in the metanephros (functional kidney) at hatching until at least 6 weeks of age and appears as wedge-shaped foci of immature renal tissue particularly beneath the capsule. These epithelial structures enlarge and become neoplastic. The supporting stroma of mesenchymal elements also proliferates and, in turn, may be altered. There is extensive multiplication of tumor cells (usually convoluted tubules and/or stroma) and varying degrees of differentiation, some abnormal. In the most differentiated form, nephrogenic cells form glomeruli, tubules, or keratinized epithelium; whereas cells of the stroma form sarcomas, cartilage, and bone. Anaplasia of kidney cells can result in sheets of large epithelioid cells with almost no tubular organization. Malformed and blocked tubules result in cysts. Nephroblastomas have been induced by BAI-A strain AMV (34, 77, 541), MAV-2(N) (443), MAV-2-O (49), and subgroup J-related strain 1911 (383). Transplantable nephroblastomas have been developed (541).

Carcinomatous growths originate only from the epithelial part of the embryonal blastema and not from mesenchymal elements. Depending on the degree of anaplasia of epithelial elements, tumors formed may be adenomas, adenocarcinomas, or solid carcinomas (34). These tumors have been induced by MC29 (34), ES4 (90), and MH2 (89) virus strains and by various field isolates (216). Renal adenomas and carcinomas can be caused by slowly and acutely transforming subgroup J ALV (381, 383).

Fibrosarcoma and Other Connective Tissue Tumors

Gross. A variety of benign and malignant connective tissue tumors occur naturally, usually sporadically, in young and mature chickens, and transmission of many of these by cell-free filtrates has been demonstrated. These tumors include fibromas and fibrosarcomas, myxomas and myxosarcomas, histiocytic sarcomas, osteomas and osteosarcomas, and chondromas and condrosarcomas. The benign tumors grow slowly, are localized, and are noninfiltrative. The malignant counterparts grow more rapidly, infiltrate surrounding tissue, and may metastasize.

Fibromas arise as firm fibrous lumps attached to the skin, subcutaneous tissues, muscles, and occasionally other organs; fibrosarcomas are of a softer consistency. In the skin, they may ulcerate. Myxomas and myxosarcomas are softer and contain tenacious slimy material: They occur mainly in the skin and muscles. Histiocytic sarcomas are firm, fleshy tumors occurring mainly in the viscera. Osteomas and osteosarcomas are uncommon and occur as hard tumors that may arise from the periosteum of any bone. Chondromas and chondrosarcomas are rare. They occur where cartilage is present and sometimes within fibrosarcomas and myxosarcomas. Ganglioneurosarcoma was reported associated with subgroup J ALV infection (238).

Microscopic. Fibromas in their simplest forms consist of mature fibroblasts interspersed with collagen fibers arranged in wavy parallel bands or whorls. Slowly growing tumors are more differentiated and contain more collagen and fewer cells than those growing more rapidly. Some fibromas may have edematous areas and should not be confused with myxomas and myxosarcomas. If necrosis, ulceration, and secondary infection have occurred, various inflammatory and necrotic alterations may be observed in the tumor. Inflammatory changes may be so prominent that the tumor may be confused with a granuloma.

Aggressive and destructive growth, their cellular composition, and the immaturity of constituent cells (Fig. 15.42) characterize fibrosarcomas. Large irregular and hyperchromic fibroblasts are abundant, and mitosis is common. Tumors contain less collagen than fibromas, and this is concentrated in and near irregular septa that subdivide the tumor. Regions of necrosis often occur in rapidly growing tumors. Edema is sometimes present. Multiple undifferentiated pulmonary sarcomas associated with subgroup J ALV infection have been reported (248).

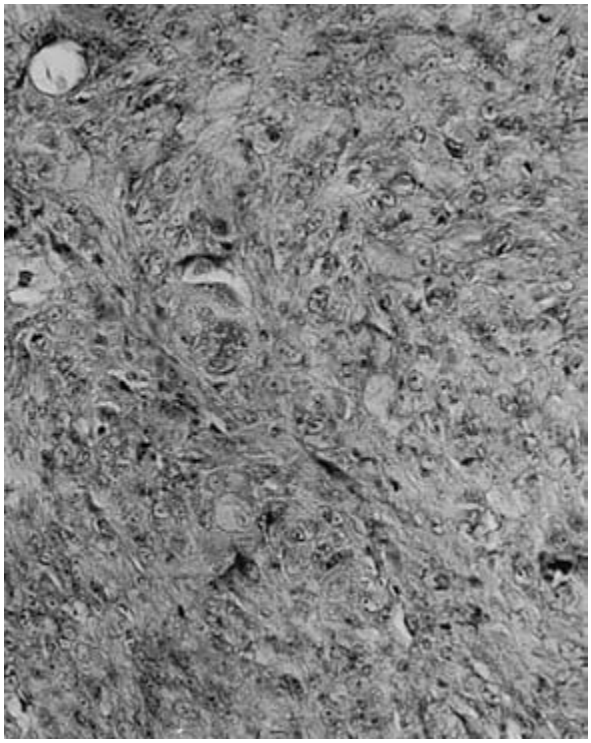
Myxomas consist of stellate or spindle-shaped cells surrounded by a homogeneous, slightly basophilic, mucinous matrix. In the malignant form (myxosarcoma), the mucinous matrix is less abundant, and fibroblasts are proportionally more numerous and immature than in myxomas (Fig. 15.43).

Histiocytic sarcomas are derived from cells of the monocyte and macrophage lineage, and the cellular constituents are highly variable both within a tumor and between tumors (Fig. 15.44). They have been reported associated with infection by subgroup J ALV (4, 247). The cells may be spindle-shaped, usually appearing in groups or bundles as in fibrosarcomas; stellate reticulum-producing elements; and/or large phagocytic cells or macrophages. Tumors apparently derived from stem cells of the myelomonocytic lineage may also be considered to be histiocytic sarcomas. The so-called endotheliomas induced by MH2 and MC29 may be tumors of this lineage (182). In primary tumors, spindle-shaped cells usually predominate; whereas in metastatic foci, primitive histiocytic forms are more numerous.

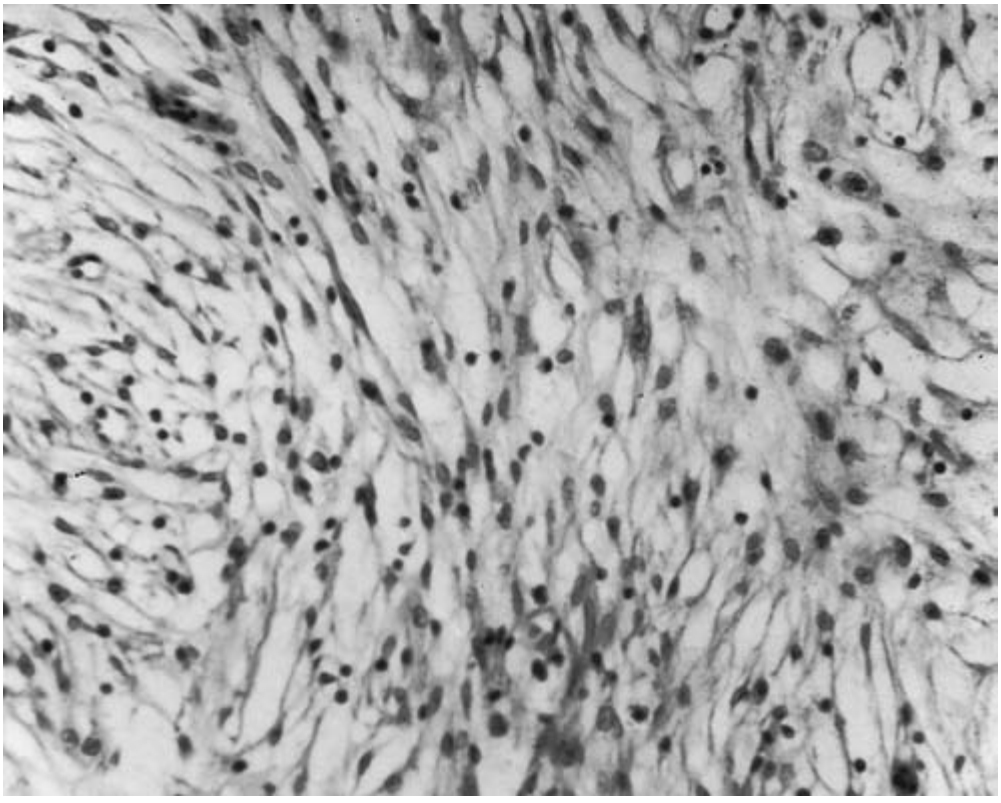
Osteomas are structurally similar to bone except that much of



15.42. Fibrosarcoma in musculature of breast. ×120. (Feldman and Olson)



15.44. Histiocytic sarcoma of heart. Note the varied character of cellular constituents. ×240. (Helmboldt)



15.43. Myxosarcoma induced by Rous sarcoma virus (RSV). ×240. (Helmboldt)

the inner histologic details is lacking. They consist of a homogenous acidophilic matrix of osseomucin containing collections of osteoblasts at irregular intervals. Osteosarcomas are usually very cellular infiltrative growths that invade and destroy surrounding tissues. The cells are spindle-shaped, ovoid, or polyhedral, and many are in mitosis. Nuclei are prominent, and cytoplasm is basophilic. Multinucleated giant cells may be quite numerous. Although very cellular and rapid growing, some areas usually have sufficient differentiation for the production of osseomucin, which is usually sufficient to identify these tumors.

Chondromas have a typical and unique structure (i.e., groups of 2 or more chondrocytes lying in a matrix of chondromucin). In chondrosarcomas, considerable cellular variation exists, ranging from the most immature to the fully mature chondrocyte.

Ultrastructural. Only the sarcomas produced by RSV have been examined in detail. The morphology of fibroblasts, macrophage-like cells, and mast cells, found in Rous sarcomas, have been described (33, 249). Tumor cells are similar to the cells in culture after infection with RSV in showing numerous pseudopodia and pronounced cytoplasmic vacuolation, which may contain virus particles (249).

Pathogenesis. Induction of sarcomas and other connective tissue tumors in the field is likely to be by activation of a cellular oncogene by a slowly transforming ALV, occurring up to several months after infection. During this process, acutely transforming viruses that carry the oncogene may be generated. Viral oncogenes that have been associated with sarcoma induction include *src*, *fps*, *yes*, *ros*, *eyk*, *jun*, *qin*, *maf*, *crk*, *sea*, and *erb*B (105, 181, 535) (Table 15.6). Experimentally, viruses carrying such oncogenes can induce sarcomas within a few days. Whether these acutely transforming viruses contribute to sarcoma induction naturally in the field is not clear. Most virus strains that induce tumors of connective tissue, whether slowly or acutely transforming, are multipotent, inducing a variety of tumors. Multipotency may depend on infection of particular target cells or on multipotency of precursor cells. Recently, an ALV related to MAV-1 was isolated from a field outbreak of sarcomas in commercial layers; inoculation of susceptible white leghorn chickens with new isolate resulted in sarcomas and myelocytomas (576).

These viral oncogenes reflect the cellular oncogenes that are activated by insertional mutagenesis and that may undergo mutation. These cellular oncogenes control a variety of functions in the cell (their products are generally growth factors, growth factor receptors, signal transducers, or DNA transcription factors), and it is their altered expression that results in the loss of regulation of cell proliferation or differentiation that causes neoplasia.

Osteopetrosis

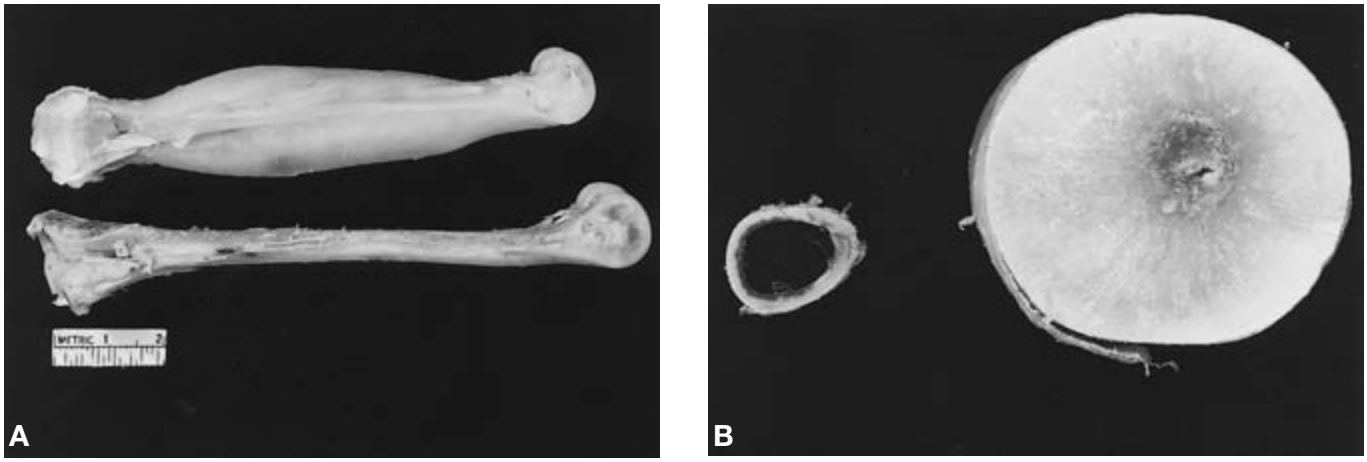
Gross. The first grossly visible changes occur in the diaphysis of the tibia and/or tarsometatarsus. Alterations soon are seen in other long bones and bones of the pelvis, shoulder girdle, and ribs but not the digits. Lesions are usually bilaterally symmetric; they first appear as distinct pale yellow foci against the gray-white translucent normal bone. The periosteum is thickened, and the abnormal bone is spongy and at first easily cut. The lesion is commonly circumferential and advances to the metaphysis, giving the bone a fusiform appearance (Figs. 15.45 and 15.46). Occasionally, the lesion remains focal or is eccentric. Severity of the lesion varies from a slight exostosis to a massive asymmetric enlargement with almost complete obliteration of the marrow cavity. In long-standing cases, the periosteum is not as thickened as it was earlier; when it is removed, the porous irregular surface of the very hard osteopetrotic bone is revealed.



15.45. Osteopetrosis. A 24-week-old chicken, injected with RPL12 at 1 day of age, with advanced osteopetrotic lesions of the shanks. (Sanger)

Early in the disease, the spleen may be slightly enlarged. Later, severe splenic atrophy occurs as well as premature bursal and thymic atrophy. LL often occurs in individual birds with osteopetrosis.

Microscopic. The periosteum over the lesion is greatly thickened from an increase in number and size of basophilic osteoblasts. The number of osteoclasts per tibia increases, but the density of osteoclasts (i.e., the number per unit volume of bone) decreases (455). Affected bones differ from normal bones in the following ways. Spongy bone converges centripetally towards the center of the shaft (Fig. 15.47). An increase occurs in size and irregularity of the haversian canals, as well as an increase in number and size, and an alteration in position, of lacunae. Osteocytes are more numerous, large, and eosinophilic; the new bone is basophilic and fibrous.



15.46. Osteopetrosis of tibia in 10-week-old chicken. A. Shorter length of bone is due to reduced growth. Lower tibia is from control bird of same age. B. Cross-section of middle of shaft of bones in A. (447)

The blood picture is ordinarily aleukemic, and a secondary anemia often exists. There may be active erythropoiesis in remaining bone marrow and sometimes in focal areas in the liver. Experimentally, viruses that cause osteopetrosis can induce an aplastic anemia and an increased corpuscular fragility (258, 406).

Ultrastructural. Virus particles bud transiently from osteoblasts and continuously from osteocytes and accumulate in the periosteocytic space. With calcification of the bone, the particles become incorporated in the bone trabeculae. No virus production is observed from osteoclasts (213).

Pathogenesis. Osteopetrosis is a polyclonal disease of the bone and is thought to be caused by high levels of virus infection perturbing the growth and differentiation of osteoblasts. Much higher levels of virus infection were found in diseased bones than in cultured osteoblasts infected with the Br21 strain of an osteopetrosis-inducing ALV (212). Severe cases of osteopetrosis contained 10 times more viral DNA, 30 times more gag precursor protein, and 2–3 times more env protein than the infected osteoblast cultures. Apparently, the infected cultures lacked aspects of the bone environment that support both the high levels of infection and the aberrant function of osteoblasts characteristic of ALV-induced osteopetrosis. The osteopetrotic lesion is basically proliferative or hypertrophic (87, 447) and may be neoplastic (54, 455). Lesions of the lymphoid organs and bone marrow are degenerative or anaplastic (258). The propensity for certain ALVs to induce osteopetrosis depends on sequences in the *gag-pol-5′env* region of the viral genome (458). Env proteins have also been implicated in osteopetrosis induction (280).



15.47. Osteopetrosis. Cross-section of humerus from 8-week-old chicken. Six separate osteopetrotic foci are present, two of which extend from endosteum to periosteum. ×18. (447)

Other Tumors

Apart from renal tumors, epithelial tumors caused by ALV are uncommon. They mainly have been reported following experimental infections with acutely transforming viruses, although some have occurred in natural and experimental infections with subgroup J ALV. Strains BAI-A (34) and HPRS-103 (383) of ALV have induced thecomas and granulosa cell tumors of the ovary. A seminoma in the testis occurred in a bird inoculated with strain MH2 (34) and possibly in birds inoculated with subgroup

J ALV isolates (394). Adenocarcinomas of the pancreas have been induced in chickens by strains MC29, MH2, and HPRS-103 of ALV (34, 339, 383). The Pts-56 strain of osteopetrosis virus produced pancreatic adenomas and adenocarcinomas and duodenal papillomas in guinea fowl (292, 294, 295). Squamous cell carcinomas have been observed in a few chicks with strains MC29 and MH2 (34). The MC29 and MH2 strains have induced hepatocarcinomas (34, 313). Other epithelial tumors induced by subgroup J ALV include cholangioma and ovarian carcinoma (383).

Strains MC29 (34) and HPRS-103 of ALV (383) have been shown to induce mesotheliomas.

Immunity

Active

Under natural conditions, most chicks become infected by exogenous ALV from penmates or their surroundings and, after a transient viremia, develop virus-neutralizing antibodies directed against virus envelope antigens that rise to a high titer and persist throughout the life of the bird (437, 481). The virus-neutralizing antibodies serve to restrict the amount of virus in the bird, which in turn, will limit neoplasia, but they generally are considered to have little direct influence on tumor growth. After inoculation of birds with ALV at 4 weeks of age or older, transient viremia was detectable at 1 week and was followed by antibodies at 3 weeks and later (324). In a study of birds naturally infected after hatching, antibodies were first detected at 9 weeks of age, with a marked increase in the proportion with antibodies between 14 and 18 weeks, when 80% were positive (437). The younger the bird at infection, the longer the duration of viremia and the greater the delay to antibody. Infection at 1 day old by inoculation may lead to a permanent viremia rather than antibody production.

Antibodies against gs-antigen may also occur in ALV-infected birds, but these apparently have no influence on tumor growth (430, 460). Antibodies against RT also have been detected in virus-infected and virus-free chickens (240).

Knowledge of the occurrence of, and the part played by, cell-mediated immunity in ALSV infections is still incomplete, but it is likely to be directed against both the virus infection and tumor formation. The presence of cytotoxic lymphocytes against viral envelope antigens has been shown in birds immunized with ALV or RSV (31, 32, 305), and cell-mediated immunity and the MHC complex are clearly implicated in the regression of Rous sarcomas (452, 453). Viral proteins expressed on the surface of tumor cells appear to be important targets for the cell-mediated immunity, and nonvirion transformation-specific cell surface antigens may also be implicated. Recently, Thacker and coworkers (511) have developed a new system for studying MHC-restricted cytotoxic lymphocyte responses to ALV infection, which should be of value in determining the role of this type of cell-mediated immunity in ALV infections. Whether cell-mediated immune responses are directed against tumor cells in lymphoid and other forms of leukosis has yet to be determined. Reviews of cell-mediated immune responses to ALSV infections have been provided by Wainberg and Halpern (538) and Schat (453).

Chickens that are infected congenitally by ALV do not develop immune responses to the virus. Instead, they become immunologically tolerant to the virus and develop a persistent viremia in the absence of neutralizing antibodies (332, 437). Inoculating chickens up to 2 weeks of age with ALV may also induce tolerant infection. Early infection with subgroup J ALV is particularly likely to induce a tolerant infection (206, 562, 564). Birds with a tolerant viremic infection are more likely to develop neoplasms than are immune-infected birds, because of the greater virus load in viremics.

Infection by ALV can depress primary and secondary antibody responses and cell-mediated immunity (439) to unrelated antigens, although these effects have been variable in different studies. Fadly *et al.* (200), in a study of congenital infection with an A subgroup ALV, RAV-1, failed to detect effects on B- and T-cell function during the early and late stages of infection, and they reported no histological damage to the bursa, thymus, or spleen. In contrast, subgroup B ALVs have been reported to induce a marked suppression of the humoral immune response to several antigens and decreased responsiveness to several mitogens (545). Evidence that subgroup J ALV is immunosuppressive appears to be equivocal at present (310, 493, 494, 574).

Passive

Serum antibodies, which are mainly in the IgG fraction (333), are passed on by the hen to her progeny via the egg yolk and provide a passive immunity that lasts 3–4 weeks. Passive antibody delays infection by ALV (565), reduces the incidence of viremia and shedding of ALV (188) and reduces the incidence of tumors (63). Level and persistence of antibody in the chick are related to the titer of antibody in the dam's serum.

Genetic Resistance

Two levels of genetic resistance to leukosis or sarcoma virus-induced tumors are recognized: cellular resistance to virus infection and resistance to tumor development (13, 27, 114, 399).

Inheritance of cellular resistance to infection is of a simple Mendelian type (Table 15.13). Independent autosomal loci control responses to infection by ALSVs of subgroups A, B, and C and are designated *tva* (tumor virus A subgroup), *tvb,* and *tvc* respectively (117). A new gene nomenclature system recently has been adopted by the Poultry Committee of the United States Department of Agriculture National Animal Genome Research Program. The new terminology is given alongside the old in Table 15.13. The *tvb* locus also controls responses to subgroup D virus (371), and linkage occurs between *tva* and *tvc* loci (174, 391). At each *tv* locus, alleles for susceptibility and resistance exist that are designated tva^s, tva^r; tvb^s, tvb^r; and tvc^s tvc^r, respectively, and the susceptibility alleles are dominant over the resistance alleles. These genes usually are abbreviated to a^s, a^r, etc. It is probable that multiple alleles occur at each locus, encoding different levels of susceptibility (173).

Inheritance of resistance to subgroup E ALV is more complex, with involvement and interaction of genes at 2 autosomal loci designated *tve* and i^e (392). A dominant resistance gene, I^e, acting epistatically, blocks susceptibility conferred by presence of e^s

Table 15.13. Genes controlling cellular susceptability to leukosis and sarcoma viruses.

	Locus		Alleles		
Virus subgroup	**Old**	**New**	**Old**	**New**	**Dominant trait**
A	*tva*	*TVA*	tva^s tva^r	TVA*S TVA*R	Susceptibility
B and D	*tvb*	*TBV*	$tvb^{s1,}$$tvb^r$	TVS*S1, S3 TVB*R	Susceptibility
C	*tvc*	*TVC*	tvc^s tvc^r	TVC*S TVC*R	susceptibility
E	*tved*	*TVE*	tve^s tve^r	TVE*S TVC*R	Susceptibility
	ie		I^e i^e		Resistance

Note: The locus designation is adapted from Crittenden (117). The new locus designation is that agreed by the Poultry Committee of the USDA National Animal Genome Research Program, 1994. The allele previously designated tvb^{S2} now is considered to be identical to tvb^{S1}. The existence of a *tve* locus is not settled. The i^e locus is now considered to be an *ev* locus, with blocking of the subgroup E virus receptor by endogenous virus ENV glycoprotein expression.

allele. It has been reported, however, that susceptibility alleles at the *tvb* locus are required for susceptibility to E subgroup virus, and until recently it was not clear whether a separate *tve* locus exists (136, 369, 370). Recent studies indicated that mutations in the *tvb* receptor gene could explain some of the reasons for the resistance to subgroups B, D or E infections (27, 296, 298). Studies suggest that the i^e locus is, in fact, an *ev* locus, the ENV glycoproteins of which block the E subgroup receptor (425). Genetic resistance to infection by subgroup J virus has not been recognized in chickens, although a number of other avian species are resistant (381, 389). Recently the specific cell receptor for ALV subgroup J was shown to be the Na+/H+ exchange type I molecule (91). Susceptibility genes such as a^s code for the presence of subgroup-specific virus receptor sites on the cell surface, which interact with viral envelope glycoprotein and allow viral penetration and infection of the cell (547). Progress is being made in identifying the nature of such receptors (see "Virus Replication"). Cells resistant by virtue of the presence of a resistance gene such as a^r in the homozygous state are believed to lack the specific receptor sites necessary for infection to occur, although nonspecific adsorption of virus to such cells can occur, but without infection being established.

Cellular susceptibility phenotypes associated with these genes are designated according to a convention that recognizes the virus subgroups to which the chicken (C) cell is resistant (/) (e.g., C/AE denotes a cell resistant to A and E subgroups but susceptible to B, C, D, and J subgroups); C/0 denotes a cell resistant to no subgroup (i.e., susceptible to A, B, C, D, E, and J).

Resistance or susceptibility conferred by these genes is expressed by all cells, whether by cells cultured *in vitro*, such as chicken embryo fibroblasts; by chicken embryo cells, such as those of the CAM; or by chickens after hatching. These responses are applicable to ALSVs sharing the same envelope glycoproteins, and, thus, viral subgroup, but most genetic studies are undertaken with appropriate subgroups of RSV, because infection of the cell is expressed within a few days by visible growth of tumor cells. Thus, the phenotype of an individual may be determined by inoculation of a standard dose of RSV into chicken embryo fibroblasts in culture, with the production of foci of transformed cells in susceptible embryo cells but not in resistant cells (434). Similarly, RSV can be inoculated onto the CAM of the chicken embryo, with or without the production of tumor pocks (129) or intracranially into day-old chicks, with death or survival as the response criterion (544) (see "Prevention and Control Procedures"). The phenotype of individual birds may be determined by culturing fibroblasts from plucked pin feather pulp and by challenging these cultures with RSV (135, 388, 487).

Genetically resistant chicks are resistant to infection and tumor induction by ALSVs of the subgroups concerned, and they usually fail to develop antibodies (118, 128). Genetic resistance to tumor development has been studied mainly with the Rous sarcoma (505), regression of which is determined by a dominant gene, *R-RS-1*, that lies within the major histocompatibility complex (MHC locus) of the chicken and located in the BBL region (107, 229, 405, 454). Conserved peptide motifs of the RSV proteins that bind to the MHC have been identified and shown to be immunoprotective against Rous sarcoma growth in chickens with class I allele B-F12 (259), and peptide motifs of the single dominantly expressed class I molecule explain the MHC-determined responses to RSV (540). The MHC (*Ea-B*) locus also influences incidence of erythroblastosis and, to a lesser extent, LL (18). Some influence of the lymphocyte antigen *Bu-1* locus on Rous sarcoma regression and of the *Th-1* locus on LL is reported (14).

Genetic resistance to LL tumor development, such as in RPL line 6, is conferred by bursal cells, not by other cellular elements of the immune system, such as thymic or thymus-derived cells or nonlymphocytes. It appears as though the intrinsic inability of the bursal target cell to become infected or transformed is the major factor in resistance (413). No obvious difference in the pattern of bursal infection in tumor-susceptible and -resistant lines was detected by Baba and Humphries (9).

Diagnosis

Isolation and Identification of Causative Agent

Because ALV is widespread among chickens, virus isolation and the demonstration of antigen or antibody have limited or no value in diagnosing field cases of lymphomas. However, assays for the

detection of ALV are very useful in identification and classification of new isolates, safety testing of vaccines, and in testing pathogen-free and other breeder flocks for freedom from virus infection. Samples most commonly used for detection of ALV include blood, plasma, serum, meconium, cloacal and vaginal swabs, oral washings, albumen of eggs, embryos and tumors (132, 193, 202, 208, 486). Virus also can be isolated from albumen of newly laid eggs or the 10-day-old embryo of eggs laid by hens that are transmitting virus vertically (483), from feather pulp (490), and from semen (456). All ALSVs are very thermolabile and can be preserved for long periods only at temperatures below −60°C. Thus, materials used for biological assays for infectious virus should be collected and placed on melting ice or stored at −70°C until assayed. In contrast, samples for detection of ALV gs antigens by direct assays can be stored at −20°C; see review by Fadly and Witter (208).

Because most strains of ALV produce no visible morphologic changes in cell culture, assays for ALV are based on the following: a) detection of specific proteins or glycoproteins coded for by 1 or more of the 3 major genes of ALV, namely *gag*, *pol*, and *env* genes (Fig. 15.22), or b) detection of specific proviral DNA or viral RNA sequences of ALV by the polymerase chain reaction (PCR) and reverse transcription (RT)-PCR, respectively.

The presence of virus is determined by the detection of ALV p27 by indirect biologic assays, such as complement fixation (CF) for avian leukosis (COFAL) (208), ELISA for ALV (125, 208), phenotypic mixing (363), resistance-inducing factor (359), and non-producer cell activation (422). Of all such assays, ELISA-ALV is the most commonly used test. All these biological assays require the use of chicken embryo fibroblasts (CEFs) with specific host range. The phenotype of CEFs most commonly used for the detection of ALV is shown in Table 5.10. Furthermore, Crittenden *et al.* (120) described the use of a Japanese quail cell line transformed by the envelope-defective Brian high titer strain of RSV [R(-)Q] for assay of exogenous and endogenous ALV. CEFs that are resistant to infection with endogenous ALV (C/E) are desirable to use in tests for detection and isolation of exogenous ALV. Other cells, such as those resistant to subgroup A (C/A) and resistant to subgroup J ALV (C/J) (265), can also be used to confirm the subgroup of isolated ALV. Testing samples on CEFs that are susceptible to all subgroups of ALV (C/O) and those that are resistant to subgroup E (C/E) can be used in differentiating exogenous and endogenous ALV. If a positive test is obtained from using C/O but not C/E CEFs, the sample is positive for endogenous ALV. Positive tests using both C/E and C/O indicate the presence of exogenous ALV. Recently, a flow cytometry method using a highly specific alloantibody termed R2 has been described for detection of endogenous ALV envelope in chicken plasma (12, 17). It should be noted that some tests such as CF and ELISA and possibly non-producer NP, PM, R(-)Q cell, and FA can be suitable for all leukosis and sarcoma viruses. The resistance-inducing factor (RIF) test can be performed only on ALVs that are not rapidly cytopathogenic. Other tests are specific for certain virus strains. Rapid transformation of fibroblast cultures is produced only by certain RSV and of hematopoietic cell cultures only by defective ALV. The test for adenosine triphosphatase activity is specific for avian myeloblastosis virus. The procedures that are most widely used have been reviewed (193, 208).

Resistance-Inducing Factor Test

In general, most ALVs do not induce alterations in cultured cells except after prolonged passage. When CEFs are infected with an ALV, however, they become resistant to superinfection by a sarcoma virus of the same subgroup. Only viruses of the same subgroup interfere with one another in this way. The property of interference has been used for assay of ALVs by the RIF test (433) and also in delineating virus subgroups (536). In the RIF test, known susceptible chicken embryo fibroblast cultures are inoculated with material suspected of containing an ALV. Cells are subcultured at least 3 times at 3–4-day intervals, and at each passage, a sample of the cells is tested for susceptibility to RSVs of different subgroups. Alternatively, supernatant fluids may be transferred to new cell cultures every 4 days. In this case, cells may be challenged without subculture 4–6 days postinoculation. Control cultures infected with known ALVs and uninfected cultures are always included to establish validity of the tests. Presence of ALV in a cell culture is indicated by a 10-fold or greater reduction in number of foci produced by a standard stock of RSV when compared with the number of foci on similarly challenged control cells. Several different challenge viruses, one for each subgroup, must be used to detect ALVs belonging to different subgroups; each requires a separate cell culture plate for testing.

Tests for Viral-Internal, Group-Specific Antigens

Detection of the major antigen (p27) present in the core of ALSVs forms the basis of several diagnostic tests for virus.

The COFAL test can be used to detect the gs antigen in cultures of fibroblasts that have been inoculated with virus (449). Cells must be susceptible to infection with the virus sought; to obtain a suitable antigen from low-titer inoculate, inoculated fibroblasts must be cultivated for 14 days before they are harvested. The harvested cells are adjusted to a standard concentration, frozen, thawed, and then used as antigen in the test. Various controls are necessary, including uninoculated fibroblasts, because these may contain gs antigen derived from endogenous ALV. Titration of complement-fixing activity of extracts of the control and inoculated cultures allows differentiation between endogenous and exogenous viral antigen, because the titer of the latter is much higher. If available, fibroblasts that do not express endogenous antigen may be used.

Because of the difficulty in distinguishing between gs antigens of endogenous and exogenous viruses, direct CF tests of infected materials, without tissue culture passage, are of limited value. Nevertheless, direct tests may be done in certain circumstances (e.g., on egg albumen) (see “Eradication”).

Complement-fixing antiserum against gs antigen can be obtained from hamsters bearing sarcomas induced by RSV (the Schmidt-Ruppin strain usually is used) (449). Rabbit and other mammalian antiserums prepared against purified gs antigens de-

rived from avian myeloblastosis virus can also be used (465, 497, 498). Antiserums have also been raised in pigeons bearing RSV-induced tumors (448, 451).

Highly sensitive radioimmunoassay (184, 446) and ELISA tests (102, 467) for gs antigens have been developed. They may be used directly for the assay of test material or indirectly using cell cultures inoculated with test material. These antigens may also be detected in cells by FA techniques (288, 373). Using indirect FA tests, monoclonal antibodies to ALV-J proved useful in the detection of ALV-J infected cell cultures (205, 417, 529). A variety of samples can be tested by ELISA for the presence of ALV; however, serum has been shown to be unsuitable for the detection of exogenous ALV by direct ELISA (385). For the detection of exogenous ALV, samples are inoculated on CEFs that are genetically resistant to subgroup E ALV. Seven to nine days later, cell lysates are tested for the presence of ALV gs antigen by ELISA (194, 208, 467). Rabbit anti-p27 antibody, which is used to coat ELISA plates, and rabbit anti-p27 conjugate, as well as complete kits for running ELISA for detection of ALV gs antigen, are available commercially.

Tests Based on Phenotypic Mixing of Viruses

CEFs can be infected with envelope-defective strains of RSV (e.g., BH-RSV) to produce transformed cells that are nonproducers of infectious RSV of subgroups A, B, and D. Superinfection of a culture of NP cells by a leukosis helper virus results in production of infectious RSV, which is detectable in supernatant fluid by assays in susceptible CEF cultures and forms the basis of the NP cell activation test (421). Several variants of the test are described. Non-producer cell activation is an example of phenotypic mixing of viruses (see "Etiology"). Non-producer cells from embryos with endogenous subgroup E ALV may spontaneously produce a subgroup E RSV from complementation of the defective RSV by subgroup E envelope. In assaying for RSV production following activation, it is then necessary to use fibroblast cultures resistant to subgroup E but susceptible to subgroups A, B, C, D, and J (C/E cells).

A useful modified NP cell activation test uses Japanese quail cells that have been transformed with envelope-defective BH-RSV. Those nonproducing R(-)Q cells can be activated to produce infectious RSV by cocultivation with C/E cells infected with the exogenous ALV under test, thus providing the R(-)Q cell test (120).

Another variant of the NP cell test is the PM test (363, 411), for which cultures of C/O fibroblasts (i.e., cells susceptible to all virus subgroups) pretreated with DEAE dextran are infected heavily with subgroup E RSV(RAV-0), producing RSV-transformed cells. About 24 hours later, the supernatant fluid is discarded, and cultures are infected with test material. Cultures are incubated for 7 days, and the culture fluid is harvested, frozen, thawed, centrifuged, and assayed for infectious RSV on C/E fibroblasts. Because E subgroup RSV is excluded, the presence of RSV foci indicates presence of exogenous ALV in the test material. Various controls must be included in the test.

Variants of these tests have also been developed to detect infectious endogenous ALV, as well as expression in chick cells of subgroup E envelope glycoprotein termed chick helper factor (chf) encoded by the endogenous viral genome (120).

Comparison of Tests

In vivo and *in vitro* cell culture tests for detection or assay of exogenous ALVs are compared in Table 15.14.

All five *in vitro* tests require a standard source of chicken embryos free from exogenous ALSVs and of known phenotype for use in cell culture. The following reagents are also required: for the RIF test, stocks of challenge RSV of each subgroup; for the COFAL and ELISA tests, specific antiserum; for the NP test, quantities of NP cells; and for the PM test, stocks of RSV with endogenous helper, RSV(RAV-0). Cells obtained from embryos of unknown genetic origin should not be used in RIF, COFAL, and indirect ELISA tests because the results may be confused by genetic resistance. Both COFAL and indirect ELISA tests require either prolonged maintenance of culture or several subcultures to propagate the virus sufficiently; therefore, much more work is involved than in the NP or PM tests.

The indicator systems are different in the 5 tests. In the RIF test, the number and appearance of RSV foci after challenge are highly dependent on the physiologic condition of the cells. Thus, when cell cultures are not in optimal condition, a RIF test cannot be performed. A cell extract is used in the COFAL and ELISA tests, however, and it can be stored frozen and tested on more than one occasion if necessary. Similarly, in the NP and PM tests, the supernatant fluid of cell cultures can be stored and tested for virus by the cell culture technique. Results are usually more clear-cut in the NP and PM tests than in the RIF, COFAL, or ELISA tests. ALV infectivity and titer could be determined by ELISA within 7 days after cultivation, but after 19 days and 3 subcultures by the RIF test (522).

The subgroup of an infecting ALV can be determined by any of the tests. In the RIF test, only RSV belonging to the same subgroup as the ALV is subjected to interference. In COFAL and ELISA tests, genetically resistant cells can be used; thus, an ALV of subgroup A will not produce CF antigens in cells of the C/A phenotype (resistant to subgroup A viruses). In the NP test, genetically resistant NP cells can be prepared, and in the PM test, genetically resistant cells can be used in the mixing phase. In NP and PM tests, supernatant from the activation or mixing phase, which contains RSV of the same subgroup as the ALV, can be placed on genetically resistant cells or embryos or used in an interference test with an ALV of known subgroup.

Immunohistochemical Tests

Direct (288) and indirect (373) FA tests as well as flow cytometry (264, 265) have been used to detect viral antigen in CEF cultures; flow cytometry has also been shown to be a very useful tool in identifying the subgroup of ALV strains contaminating commercial Marek's disease vaccines (187).

When mammalian gs antiserums are used and cells are fixed in acetone, the test becomes analogous to the COFAL test. Avian serums are subgroup specific or even type specific (373). Other immunohistochemical techniques have also been described (166, 185, 233, 234).

Table 15.14. Comparison of methods for assaying exogenous avian leukosis viruses.

Method	Requirements	Response measured	Additional requirements for subgroup determination	Time required (days)[c]
In vivo				
Chick inoc 1 day IA	LL susceptible[a]	LL	Genetically resistant chickens	270
Chick inoc 1 day IA	Erythro susceptible[b]	Erythro	Genetically resistant chickens	63
Embryo inoc 11 days IV	Erythro susceptible	Erythro	Genetically resistant chickens	43
Cell culture	RSV pseudotypes, C/E cells	Resistance to formation of RSV foci in CC[d]	Challenge virus of known subgroup	12 + 6
RIF				
COFAL	Hamster antiserum, C/E cells	Complement fixation	Genetically resistant cells	14 + 1
ELISA	Enzyme-linked antisera C/E cells	Color change of substrate	Genetically resistant cells	14 + 1
NP	NP cells (chicken or quail)	RSV foci in CC	Genetically resistant cells of RIF test with leukosis virus of known subgroup	8 + 6
PM	RSV (RAV-0), C/O, and C/E cells	RSV foci in CC	Genetically resistant cells of RIF test with leukosis virus of known subgroup	5 + 6

Note: C/E, cells, genetically resistent to infection with viruses of E subgroup, but susceptible to viruses of other subgroups; C/O, cells phenotypically susceptible to infection by viruses of all subgroups; COFAL, complement fixation for avian leukosis viruses; CC, cell culture; ELISA, enzyme-linked immunoabsorbent assay; erythro, erythroblastosis; IA, intraabdominal; IV, intravenous; LL, lymphoid leukosis; NP, nonproducer; RIF, resitance-inducing factor; RSV, Rous sarcoma virus; RSV (RAV-0), Rous sarcoma virus with endogenous helper.

[a] Chickens susceptible to LL tumor formation (e.g., line 15I chickens).

[b] Chickens susceptible to virus infection and to development of erythroblastosis (or myeloblastosis).

[c] Approximate number of days necessary to cultivate the virus plus the number of days to indicate the presence of virus.

[d] Cell culture.

Enzyme Assays

Avian myeloblastosis virus has on its surface an enzyme (ATPase) that dephosphorylates adenosine triphosphate. This activity can be used as a quantitative assay to determine the amount of virus present in the plasma of infected chickens or in supernatants of myeloblast cultures (38).

Assays for RT activities have been used for the detection of oncogenic RNA viruses including all ALSVs (507). Detection of this enzyme, either directly when the correct template is used (287, 510) or indirectly when the radioimmunoassay is used (368), is an indication of presence of virus. Most recently, a highly sensitive PCR-based RT assay has been used to screen human vaccines that are produced in CEF or embryonated eggs for freedom from avian retroviruses (267, 519). In all tests for detection of ALV by RT assay, control tests must be conducted to rule out the presence of RT and of other retroviruses.

Detection of Viral Nucleic Acids

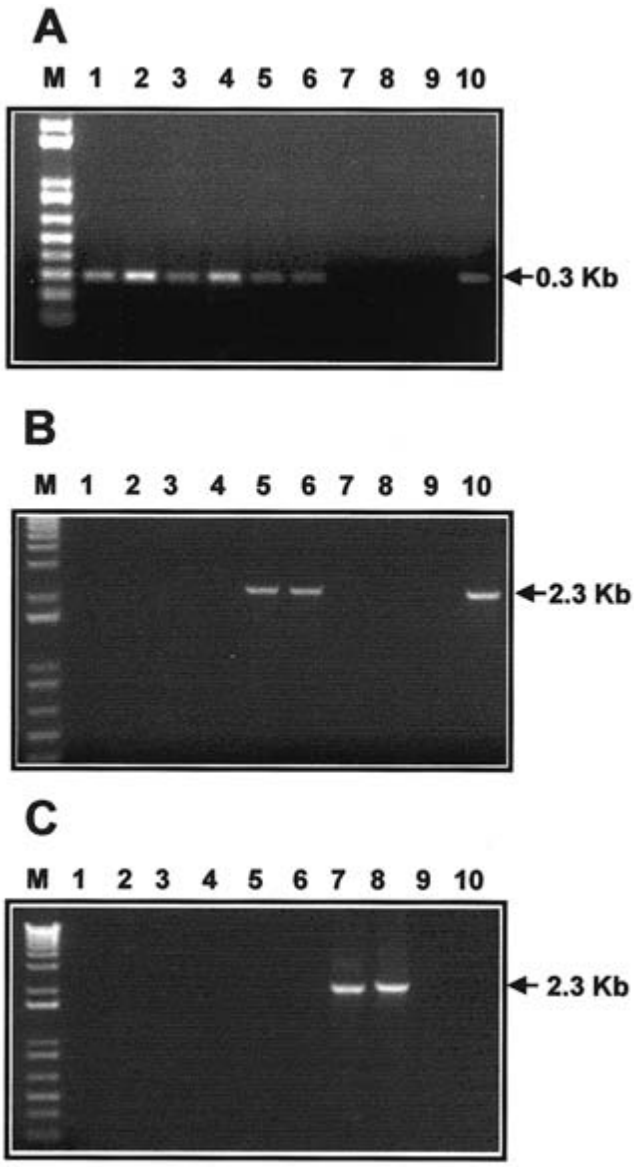
Blot-hybridization analysis of viral DNA or RNA in cell extracts is used increasingly for the detection of virus in avian tumor virus research (553, 554). The PCR is the most common DNA-based test used for detection and identification of ALV including subgroup E viruses (Fig. 15.48). RT-PCR has also been used to detect several subgroups of ALV (254). Most sequences used for developing primers are located in the env and LTR regions (see previous discussion). A specific PCR for ALV subgroup A can be used to detect proviral DNA and viral RNA in various tissues from ALV-infected chickens (526). Reverse transcriptase nested PCR (RT-nested PCR) test that amplifies a fragment of the LTR of exogenous ALV subgroups A, B, C, D and J, but not endogenous retroviral sequences has been described (225). Several primers specific for the detection of the most commonly isolated ALVs, particularly subgroup A (322), and the new subgroup ALV-J (462, 476, 477) have been developed. Other primers specific for endogenous, subgroup E ALV can also be used to detect cell culture infected with endogenous ALV-E, but not those infected with exogenous ALV of subgroups A, B, C, D, and J (208). PCR tests have been used to detect and characterize ALV strains contaminating Marek's disease vaccines (187, 573).

Hematopoietic Transformation

Avian myeloblastosis virus, an acutely transforming strain of ALV, harboring an oncogene, can infect and transform cultures of avian myeloblasts. Assays usually are based on a quantal response in which individual cultures are scored as positive or negative (23, 341). Focus assays for myeloblastosis, erythroblastosis, and other defective ALVs have been developed (241, 243, 343). Cultured chicken bone-marrow cells and blood monocytes are useful in isolation and propagation of acutely transforming viruses recovered from cases of myeloid leukosis induced by strain HPRS-103 ALV-J (384).

Transformation of Fibroblasts and Cytopathology

Avian sarcoma viruses transform spindle-shaped flat CEFs into spherical and refractile foci (414, 508) that can be seen microscopically after 4–5 days (Fig. 15.26). Genetically susceptible



15.48. PCR analysis of DNA isolated from line 0 CEF uninfected and infected with RAV-1 (ALV-A), RAV-2 (ALV-B), RAV-49 (ALV-C), RAV-50 (ALV-D), ADOL-HC-1 (ALV-J), and ADOL-R5-4 (ALV-J), and 15B1 cells uninfected and infected with RAV-0 (ALV-E) and EV21 (ALV-E). A. PCR analysis using primers specific for ALV-A-E (H5/AD1; reference 400). B. PCR analysis using primers specific for ALV-E (H5/DSW7.3; reference 400; reference 257). C. PCR analysis using primers specific for ALV-J (6J/S2; reference 385; reference 399). Lanes: M, 1 Kb plus DNA ladder; 1, RAV-1; 2, RAV-2; 3, RAV-49; 4, RAV-50; 5, RAV-0; 6, EV21; 7, ADOL-HC-1; 8, ADOL-R5-4; 9 line 0 CEF; 10, 15B1 CEF. (Lupiani)

cultures are inoculated with test material. The next day, medium is decanted and is replaced with an agar overlay (208). Inoculated cultures should be examined daily for RSV-induced foci, which usually develop within 4–7 days PI.

Sarcoma viruses also activate NP cells, produce the complement-fixing gs antigen, and can be detected by the FA technique. A number of defective acute ALVs can also transform CEFs (243). ALVs of subgroups B and D induce cytopathic effects in cell culture (242, 286). Because this property is restricted to a few viruses, it cannot be used as an assay for field viruses.

Serology

Plasma, serum, or egg yolk are suitable samples for the detection of antibodies to ALSVs.

Tests

Antibody to ALV can be measured by its reaction with RSV or ALV; a virus of one subgroup will not be neutralized by antibodies provoked by a virus of a different subgroup (536). Usually, a 1:5 dilution of heat-inactivated (56°C for 30 minutes) serum is mixed with an equal quantity of a standard preparation of RSV of a known pseudotype; after incubation, the residual virus is quantitated by any one of many procedures, the cell culture assay being most commonly used (437). A microneutralization test to assay for residual virus can be used for detection of ALV antibody (208). The test can be conducted in 96-well microtiter plates, and the neutralization of the virus is determined by an ELISA on culture fluids (199).

An indirect immunoperoxidase absorbance test (335, 336), ELISA tests (337, 472, 520, 523), and flow cytometry (264, 265) have been described for the detection of antibodies. ELISA kits for the detection of antibodies to ALV subgroups A and B are available commercially. Also, molecularly cloned, baculovirus-expressed envelope glycoproteins of ALV-J now are being used in commercial ELISA kits specific for the detection of antibody to ALV-J (314, 529).

Serotypes

Based on host range, interference spectrum, and viral envelope antigens, viruses of L/S group occurring in chickens are divided into 6 subgroups (A, B, C, D, E, and J) (378).

Viruses of different subgroups can be distinguished by the ability of monovalent antiserums to neutralize them. However, even though some cross-neutralization usually exists between viruses belonging to the same subgroup, the kinetics of neutralization vary, and slopes of curves for heterologous systems differ from those of homologous systems. No common neutralization antigens are among the viruses of different subgroups, except for a relationship between subgroups B and D. The diagnosis of infection by serologic means requires that representatives of all serotypes be employed. ALVs themselves may be used, but more commonly, RSV pseudotypes are employed in the neutralization tests (208).

Differential Diagnosis

Lymphoid Leukosis

Differential diagnosis of lymphomas in chickens can be difficult (189, 190, 196, 539). The 2 most common lymphoid neoplasms, namely Marek's disease (MD) and LL, are particularly confusing (567). Lymphoid tumors observed in REV-infected chickens, although only infrequently in cases of use of REV-contaminated vaccines, or under experimental conditions (see "Reticuloendo-

theliosis"), may also add to the confusion. LL cannot be differentiated from REV-induced bursal lymphomas on the basis of pathology, immunohistochemistry, and molecular changes in the c-*myc* region. Virologic, serologic, or PCR tests may be helpful in establishing infection for 1 virus and exclusion for the other. However, such assays are not particularly helpful in the diagnosis of virus-induced lymphomas of chickens including LL, as avian oncogenic viruses are widespread, and infection in the absence of tumor formation is common. Detection of proviral DNA (143, 189, 190) and integration junctions (96, 237) by PCR assays has been shown to be useful for tumor diagnosis.

Because LL tumors should contain ALV proviral DNA sequences inserted near the c-*myc* gene, differentiation between LL and REV-induced bursal lymphomas can be made by Southern blots and hybridization analysis of tumor DNA for clonal insertion of ALV (see previous discussion).

Lymphomas in which bursal tumors are lacking or in which the latent period is too short for that of LL can be confused primarily with MD; however, under certain circumstances, REV-induced lymphoma should also be ruled out (see "Reticuloendotheliosis"). In cases in which bursal tumors are lacking, LL and MD can be differentiated only with difficulty, because similar lymphoid tumors may occur in both diseases in the same visceral organs during the same age period. Visceral lesions of these 2 diseases cannot be distinguished by gross examination. Diagnosis is possible in most instances on careful microscopic examination; however, considerable experience is necessary. In coming to a decision, history, signs, gross and microscopic lesions, and cytology should all be considered. This section describes the points that should receive special attention (189–191, 416, 539).

Ordinarily, LL does not occur before 14 weeks of age, and most of the mortality occurs between 24 and 40 weeks. MD, however, may occur as early as 4 weeks, and the mortality peak varies from 10–20 weeks. Occasionally, losses continue and may reach a peak after 20 weeks.

Nodular tumors of the bursa can often be palpated through the cloaca in birds infected with ALV. Paralysis associated with gross lesions in autonomic and peripheral nervous systems and gross lesions of the iris ("gray eye") are specific for MD.

As stated previously, the bursa of Fabricius plays a central role in development of LL. When distinct focal or nodular lymphoid tumors are present in the bursa, a diagnosis of LL can be made; however, REV-induced bursal lymphomas must be ruled out. Such tumors are sometimes quite small and may be overlooked. In some birds, MD induces a premature atrophy of the bursa. In others, the bursa may be tumorous, in which case the walls and the plica may be thickened from interfollicular infiltration with pleomorphic lymphocytes. In contrast, intrafollicular tumors of the bursa consisting of uniform large lymphocytes are usual with LL.

Microscopic lymphoid infiltration in nerves, cuffing around small arterioles in the white matter of the cerebellum, and the feather follicular pattern of lymphoid cell infiltration in the skin, which are characteristic of MD, are not seen with LL.

Cytologically, LL tumors generally are composed of a homogeneous population of lymphoblasts (see Fig. 15.30). In contrast, tumors of MD usually contain lymphoid cells varying in size and maturity from lymphoblasts to small lymphocytes, and plasma cells may also be present. Special stains such as methyl green pyronin are helpful for cytology. Immature lymphoblasts characteristic of LL tumors are highly pyroninophilic; whereas the medium and small lymphocytes that predominate in tumors of MD do not stain with pyronin.

LL tumors are composed almost entirely of B cells and have surface IgM markers; whereas 60–90% of MD tumor cells are T cells that lack IgM markers and only about 3–25% are B cells. In addition, from 0.5–35% of MD tumor cells have a tumor-associated cell surface antigen (MATSA), which is absent from LL tumor cells (168, 355, 356, 404). Recently, Witter *et al.* (567) introduced a diagnostic strategy for the differential diagnosis of viral lymphomas in chickens.

Other diseases that may be confused with LL are erythroblastosis, myeloblastosis, myelocytomatosis, pullorum disease, tuberculosis, enterohepatitis, Hjarre's disease, and fatty degeneration of the liver.

Erythroblastosis

Although gross lesions of liver, spleen, and bone marrow provide the basis for a presumptive diagnosis, a firm diagnosis must be based on finding large numbers of erythroblasts by microscopic examination of a blood smear and sections or smears of liver and bone marrow. Chickens in early stages of disease or without obvious signs may be missed easily unless microscopic examination is made.

Erythroblastosis with concurrent anemia is often difficult to differentiate from anemia resulting from non-neoplastic causes. In erythroblastosis, there is usually a defect in maturation of erythroblasts, resulting in the presence of large numbers of them and very few polychrome erythrocytes. In anemia, the reverse usually occurs. Extramedullary erythropoiesis and stasis of erythroblasts in the sinusoids are usually more prominent in erythroblastosis than in anemia.

Erythroblastosis can be distinguished from myeloblastosis on the following grounds. In myeloblastosis, the liver is usually pale red and the marrow is whitish; whereas in erythroblastosis, the liver and marrow are usually cherry red (see Fig. 15.33B,C). In myeloblastosis, the cells accumulate intravascularly and extravascularly, whereas in erythroblastosis they are always intravascular. The erythroblast and myeloblast may be difficult to distinguish. Erythroblasts have a basophilic cytoplasm and perinuclear halo; myeloblasts often have some granules (see Fig. 15.33D,E).

Erythroblasts are cells of the erythropoietic system and can be differentiated from cells of the myelopoietic system on the basis of the presence of certain markers. Thus, erythroblasts have erythroid markers including hemoglobin, chicken erythrocyte-specific histone H5 and chicken erythrocyte-specific cell surface antigens detected by immunofluorescence. Myeloblasts and myelocytes have myeloid markers including adherence and phagocytic capacity, Fc receptors as determined by rosette formation, macrophage- and granulocyte-specific cell surface antigen as detected by immunofluorescence, and dependence of colony formation on colony-stimulating factor (243, 344).

Erythroblastosis can be distinguished from LL by the nature and distribution of lesions. Microscopically, the cytoplasm of lymphoblasts is somewhat less basophilic than that of erythroblasts, and there is also a larger nuclear-cytoplasmic ratio than in the latter cells. Lymphoblasts are more variable in size and shape than erythroblasts, but they are all at the same primitive developmental stage. Lymphoblasts tend to have an ovoid rather than spherical nucleus and a finer, more delicate-looking chromatin network.

Myelocytomas are distinguished easily from erythroblastosis.

Myeloblastosis

As in erythroblastosis, a tentative diagnosis may be based on gross lesions; however, these are often so similar to those of LL that specific diagnosis cannot be made without examination of a blood smear. Examination of liver or bone marrow sections is helpful when identity of the cell type is in doubt. The myeloblast is, on the average, smaller than the erythroblast or lymphoblast; its cytoplasm is more acidophilic and is polygonal or angular. The nucleus is less vesicular; the nucleolus, while present, is not nearly so frequently seen or conspicuous as in the other two leukoses. Myeloblasts also have physiologic markers that identify them as members of the myeloid series (see the previous discussion).

Myelocytomatosis

The distinctive character and location of tumor (see the previous discussion) provide the basis for diagnosis, which can be verified by examination of a stained smear or tumor section. Gross tumors must be differentiated from myeloblastosis, LL, osteopetrosis, and necrotic and/or purulent processes occurring in tuberculosis, pullorum disease, and mycotic infections. In recent outbreaks of ALV-J induced tumors, myelocytomatosis was diagnosed primarily on the basis of presence of characteristic microscopic feature of tumor cells (206, 381, 383, 394).

Hemangioma

Hemangiomas on the skin should be differentiated from wounds, bleeding feather follicles, and cannibalism. Those in the visceral organs should be differentiated from hemorrhages and sarcomas.

Renal Tumors

Renal tumors should be suspected when tumor nodules or large masses are found only in the kidney or are encountered suspended from the lumbar region. Diagnosis can be verified by microscopic examination. Tumors should be differentiated from other causes of kidney enlargement including hematomata, LL, and accumulation of urates.

Osteopetrosis

Bone lesions of advanced cases are sufficiently distinctive to present no difficulty in diagnosis. Cross and longitudinal sectioning of long bones is helpful in detecting slight exostoses and endostoses, particularly in early stages.

Among other osteopathies (407), rickets and osteoporosis can be differentiated from osteopetrosis by their epiphyseal formation of osteoid or porous bone. In perosis, there is twisting and flattening of the shank while the bone structure itself remains normal.

Connective Tissue Tumors

These tumors are usually easy to distinguish from the leukoses. They should not be confused with granulomas (Hjarre's disease, tuberculosis, pullorum disease), results of trauma, myelocytomas, or leiomyomas.

Intervention Strategies

Vaccination

No commercial vaccine is available for the protection of chickens from infection with ALV. However, the idea of using vaccines to increase host resistance to ALV infection is very attractive (443). In a series of attempts to inactivate ALV by various means, however, Burmester (67) demonstrated that ability of these virus preparations to induce antibody was destroyed almost concurrently with inactivation. Attempts to produce attenuated strains of ALV that do not induce disease have also failed (364). Results of experimental vaccination with live ALV on shedding and congenital transmission of the virus are equivocal. Some success has been obtained in attempts to increase the resistance of the host to RSV by immunization with viral or cellular antigens (40, 379). The use of experimental recombinant ALSVs as vaccines may prove to be a valuable adjunct to current programs for reduction or eradication of ALV infection. Recombinant ALVs expressing subgroup A (93, 192, 329, 568) and ALV-J (314, 529) envelope glycoproteins have been produced that could have potential as vaccines to protect against horizontal transmission. It is worth noting that congenitally infected chicks are immunologically tolerant and, thus, cannot be immunized even if a suitable vaccine was available. These chickens constitute the major source of ALV transmission and are the most likely to develop neoplasms.

Treatment

No practical measures have been found for treatment of the various forms of the avian leukosis complex. In general, all attempts to treat virus-induced neoplasia have resulted in negative or nonreproducible results.

Prevention and Control Procedures

Eradication

Eradication of ALV from primary breeding stocks is the most effective means for controlling ALV infection in chickens. Primary breeding companies of layer-type and meat-type stock have made significant progress in reducing or eradicating ALV of subgroups A, B, and J from their elite breeding lines.

Until 1977, eradication was only applicable to experimental or special SPF flocks because methods used were long, complicated, and expensive. Since then, eradication from commercial flocks has become feasible (92, 387, 390, 483, 566) using the techniques described by Spencer *et al.* (486).

Programs for eradication of ALV infection depends on breaking the vertical transmission of virus from dam to progeny.

Breeder hens are tested by various methods for the presence of ALV, and those that test positive are discarded. In order to establish an ALV-free flock, it is necessary to hatch, rear, and maintain in isolation a group of chickens free from congenital infection. To achieve this, embryos must be obtained from dams that are not transmitting virus to their progeny. In earlier work on development of ALV-free flocks, several methods for selecting dams were used or recommended. The dams selected to produce the next generation and hoped to be a virus-free generation were (1) immune, nonvirus shedders. Hens with antibody were selected on the assumption that they were less likely than hens without antibody to shed virus. Chicks were hatched from those that did not transmit virus to their embryos, based on tests on at least 3 embryos per hen (261). (2) Nonimmune, nonvirus shedders. Hens without antibody were selected on the assumption that they had not ever been infected and were less likely than hens with antibody to become intermittent shedders (317). (3) Nonviremic hens regardless of immune status. These were identified and used to provide replacements; however, up to 4 generations of testing were needed before flocks were free of viremics, and infection of nonviremics was not ruled out (572).

Application of eradication programs of ALV to commercial flocks has depended on associations between virus infections in hens, eggs, embryos, and chicks (486): (1) Egg albumen may contain exogenous ALV and gs antigen, and both are usually present together. (2) A strong association exists between ALV or gs antigen in egg albumen and ALV in vaginal swabs. (3) An association exists between ALV in vaginal swabs or egg albumen and ALV in chicken embryos and newly hatched chicks. Consequently, hens with a low probability of producing infected embryos are hens negative for virus (or gs antigen) by the vaginal swab test, or hens that produce eggs with albumen free from virus or gs antigen. Commonly, virus in vaginal or cloacal swabs may be detected by ELISA, NP, or PM tests and in egg albumen by ELISA or direct COFAL tests. It is unlikely that a single test will detect all potential shedder hens. A problem that arises in applying the ELISA test to albumen or swabs is the need to differentiate positive reactions due to the presence of gs antigen derived from endogenous ALV or loci from the reactions due to the presence of exogenous ALV infection (269). Reactions due to the latter are usually markedly higher, but the setting of the boundary between endogenous and exogenous virus infections is sometimes difficult and somewhat arbitrary. High reactions due to exogenous virus are clearer with albumen samples than with swabs (132). There is a prospect that monoclonal antibodies developed against p27 protein will be used in ELISAs to differentiate between endogenous and exogenous infections (315); also, PCR assays using primers based on the proviral LTR will be useful.

A procedure for eradication of ALV involves (1) selection of fertile eggs from hens negative in the egg albumen or vaginal swab test (134, 202, 360, 386); (2) hatching of chicks in isolation in small groups (25–50) in wire-floored cages, avoidance of manual vent sexing (203), and of vaccination with a common needle (147) to prevent mechanical spread of any residual infection; (3) testing of chicks for ALV by a biologic assay or PCR on blood, discarding reactors and contact chicks (202, 203, 360); and (4) rearing ALV-free groups in isolation (203, 566). In practice, selection of hens with a low shedding rate is a simpler requirement to fulfill than the subsequent chick testing and isolation rearing needed to achieve complete eradication. Consequently, some commercial breeder organizations are concentrating only on reduction of infection rate by hen testing. Progress in reducing shedding rates was reported for many lines, although some responded poorly (201, 362). Poor response to selection was not inherent in the lines but appeared to be related to environmental factors (201). For a review of these and other control methods, see Spencer (484, 487), de Boer (144), Payne and Howes (387), and Payne and Venugopal (394). Small group hatching and rearing procedures allowed identification and removal of groups containing chickens infected prior to hatching and prevented horizontal transmission of ALV-A in egg-type chickens (203) and ALV-J in meat-type chickens (566).

Chicks are most susceptible to contact infection by ALVs during the period immediately after hatching. Although congenitally infected hatchmates are likely to be the main source of such infection, several procedures can reduce or eliminate infection remaining from previous populations. Incubators, hatchers, brooding houses, and all equipment should be thoroughly cleaned and disinfected between each use. Chick boxes should not be reused, and each farm ideally should have only one age group of chickens. The danger of introducing strains of virus not already present in the population can be eliminated if eggs or chicks from different sources are not mixed, and if chicks are reared under isolation conditions that will prevent cross-contamination of flocks.

Experimental vaccination of chickens with virulent ALV at 8 weeks of age is reported to prevent virus shedding to eggs and to facilitate eradication of ALVs (420), but this could not be confirmed by Okazaki *et al.* (361). Chicks vaccinated at 8 weeks of age or older do not usually shed virus to their eggs, they may harbor it, particularly in white blood cells and the spleen (324).

Selection for Genetic Resistance

The frequencies of the alleles that encode cellular susceptibility and resistance to infection by exogenous ALSVs (see "Genetic Resistance") vary greatly among commercial lines of chickens (126, 345). In some lines, high frequencies of a resistant allele may be found naturally. In others, frequencies of the resistant alleles can be increased by artificial selection. In practice, emphasis is placed on resistance to the predominating A subgroup ALV and sometimes to B subgroup also. With the emergence of ALV-J infection in meat-type chickens, the value of developing lines that are resistance to ALV-J should be determined.

In artificial selection, genotypes of unknown parents may be determined in a progeny test by mating them to recessive tester birds of the subgroup in question (e.g., $a^r a^r$ for A subgroup virus) (372). Depending on the segregation of susceptible and resistant progeny in a particular mating, the genotype of the unknown parent may be determined. The phenotypic identification of progeny in the test may be determined by inoculation of RSV onto the CAM, the embryo being scored as susceptible or resistant on the basis of pock count (129) or intracranial inoculation of RSV into hatched chicks,

chicks being scored on the basis of death or survival (442). The former method is preferable and has many advantages.

Crittenden (115, 118) discussed some of the problems raised by this approach. Mutant viruses are more likely to overcome resistance from a single gene than that related to a multiple gene effect, and mutant subgroups then may be favored. In a host population resistant to virus penetration, there can be no effective selection for resistance to the development of neoplasms; for this reason, mutant viruses may take over. It is probable that past selection for host viability has increased the resistance of infected birds to the development of neoplasms. This type of resistance is poorly defined but may be controlled by a number of genes and is, consequently, more difficult to overcome by viral mutation. A prospect suggests that it will become possible to control ALV infections by the development of resistant stock by transgenesis (117). Recent methods of developing commercial chicken strains free of endogenous retroviruses (15) and the new methodologies of transgenesis using genetically modified primordial germ cells (524, 525) point towards the feasibility of using these approaches for generating resistant stock.

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Reticuloendotheliosis

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Introduction

Definition

Reticuloendotheliosis (RE) designates a group of pathologic syndromes in several avian species caused by retroviruses of the reticuloendotheliosis virus (REV) group. The disease syndromes include 1) a *runting disease* syndrome, 2) *chronic neoplasia* of lymphoid and other tissues, and 3) *acute reticulum cell neoplasia*. Disease manifestations are not common although infection appears to be widespread.

The laboratory-derived strain T is defective for replication in chicken fibroblast tissue cultures and possesses a unique oncogene of cellular origin (v-*rel*) that is responsible for its acute oncogenicity (101, 102). Stocks of strain T also contain a nondefective helper REV that replicates in chicken fibroblast cultures but lacks acute oncogenic properties (101). The helper virus has been variously designated as *REV-A* (101) or as *nondefective strain T* (268). The REV group now includes strain T, *chick syncytial virus* (51), *duck infectious anemia virus* (138), *spleen necrosis virus* (238). Many other nondefective strains have been isolated from a variety of avian species including turkeys, chickens, ducks, pheasants, geese and prairie chickens (259). Nondefective strains are a single serotype, but have been differentiated into 3 antigenic subtypes (45).

The nondefective REVs are responsible for the runting disease

and the chronic neoplastic disease, both of which occur in nature. The acute reticulum cell neoplasia is induced only by strain T and is not known to occur in nature.

Economic Significance

Dramatic economic loss from runting syndrome or chronic neoplasia can occur when REV-contaminated vaccines are administered to very young chickens (83, 113, 128). However, such events are rare and on the basis of reported clinical disease, the economic importance of RE in turkeys and chickens is minor. Progeny of seropositive flocks are prohibited from export to certain countries, however, causing economic loss to certain breeders. Significant costs are also incurred by vaccine companies and producers of specific-pathogen-free flocks who must routinely monitor their products for REV contamination. Potential problems such as the possibility of immunodepressive disease from environmental exposure or contaminated vaccines, or of infection becoming endemic in valuable breeding stock have raised the level of concern (257).

Public Health Significance

The extended host range of REVs, which includes certain mammalian cells (1, 250) and other characteristics that suggest an evolutionary linkage with mammalian retroviruses (14, 127, 192), raised the possibility of human infection (114). Johnson and colleagues reported the detection of antibodies against REV antigens in human sera by ELISA and Western blot assays (115, 116), but titers were low and most of the reactivity could be removed by absorption with normal chicken tissue. However, other workers have considered the evidence for human infection with REV as insufficient to warrant concern (66, 68, 93).

Scientific Significance

RE has received uncommon attention by researchers. The acute and chronic neoplastic diseases represent (with Marek's disease and lymphoid leukosis) a third etiologically distinct group of avian viral neoplasms. The virus has a wider host range than other avian tumor viruses and can infect or transform a variety of cell types (1, 14, 251). The various REV-induced neoplastic syndromes in chickens resemble both lymphoid leukosis and Marek's disease and RE is a model for the study of immunosuppression in chickens. REV has become a frequently used model system in comparative retrovirology. REV can integrate into the genome of large DNA viruses, including Marek's disease and fowl pox (100, 110). REVs have been used as expression vectors to insert foreign genes into chicken and mammalian cells; such vectors may have diverse uses from production of transgenic chickens (28) to gene therapy in humans (69).

History

The initial REV isolate, strain T, was obtained in 1957 from a turkey with visceral lymphomas and was serially passaged over 300 times in turkeys and chickens by Twiehaus and colleagues at Kansas State University with cellular and cell-free inocula. Although these authors obtained considerable experimental data on this virus during the period 1958–60, publication was delayed (195). In the meantime, Sevoian obtained the isolate from Twiehaus and found it acutely oncogenic, causing death of young chicks 6–21 days after inoculation (210). Theilen *et al.* confirmed the acute oncogenicity of strain T for young chickens, turkeys and Japanese quail; these authors were the first to designate the disease as a *reticuloendotheliosis* on the basis of the prominent cell in the neoplastic lesion which he called reticuloendotheliosis (234), now termed acute reticulum cell neoplasia. Subsequently, Bose and coworkers designated the strain T virus as reticuloendotheliosis virus (27).

Purchase (183) recognized the antigenic relationships between strain T and the previously-characterized but nononcogenic chick syncytial spleen necrosis and duck infectious anemia viruses, thus establishing the concept of a reticuloendotheliosis virus group where the member strains have diverse biological properties. Thus, nomenclature for the disease and the virus originated from the atypical pathology induced by the defective strain T and was extended to all viruses in the group, even though reticuloendotheilal cell lesions are rarely, if ever, induced by infection with nondefective strains.

The literature on REV is substantial and other reviews may be consulted for additional details (11, 26, 68, 146, 154, 178, 184, 255, 259).

Etiology

Classification

REVs are retroviruses immunologically, morphologically, and structurally distinct from the leukosis/sarcoma group of avian retroviruses (see review (184). The International Committee on Taxonomy of Viruses has recently classified REVs within the family *Retroviridae*, subfamily *Orthoretrovirinae*, genus *Gammaretrovirus* (30). REVs were formerly classified within the genus *mammalian C-type*, whereas the avian leukosis viruses are classified in a separate genus (48). The phylogenetic relationship with mammalian C-type retroviruses is supported on the basis of morphology, nucleic acid sequences, amino acid sequences of major polypeptides, and immunologic determinants (see review (153) and receptor interference patterns (123).

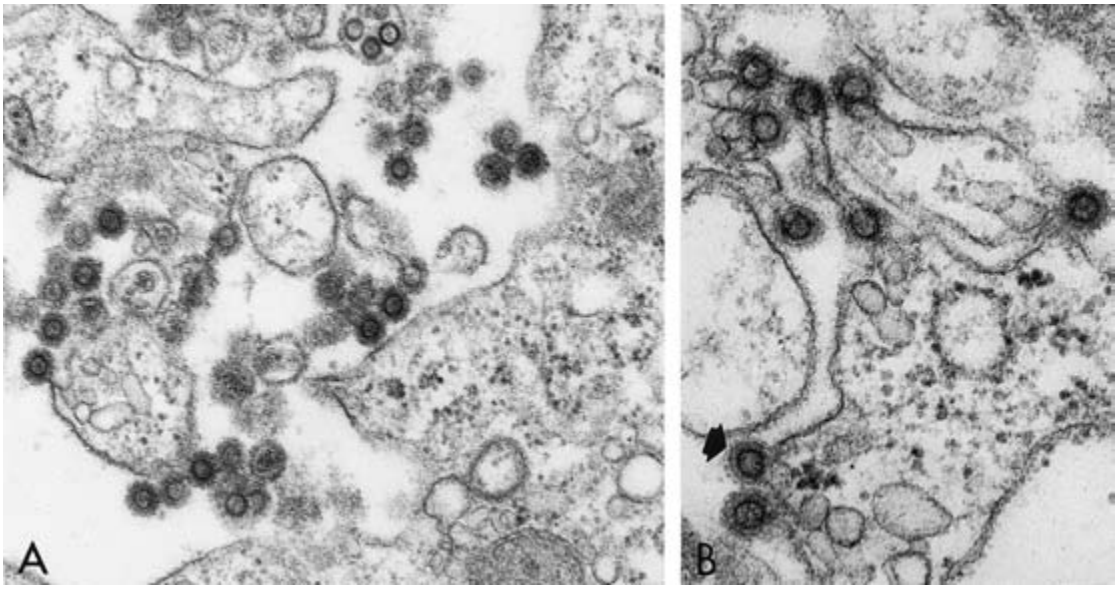
Morphology

Viral particles are typical of retroviruses; they are about 100 nm in diameter (277), and are covered with surface projections about 6 nm long and 10 nm in diameter (118). Virions have a density of 1.16–1.18 g/ml in sucrose density gradients (20), but can be differentiated from avian leukosis/sarcoma viruses by morphology in thin sections (152, 277). The morphology of the viral particles is shown in Figure 15.49.

Chemical Composition

Nucleic Acid

Genomic single stranded RNA of REVs consists of a 60–70 S complex containing two 30–40 S RNA subunits, each having a size of about 3.9×10^6 d (22, 142) . The nondefective REV has a genome of about 9.0 kb, while the replication-defective strain T genome is only about 5.7 kb due principally to a large deletion



15.49. Electron micrographs of thin sections of chicken embryo fibroblasts infected with REV. A. Typical virus particles in the extracellular spaces. ×40,000. B. REV particles budding from the plasma membrane of infected cells (arrow). ×60,000. (Nazerian)

in the *gag-pol* region and a smaller deletion in the *env* region (49). Moreover, the replication-defective strain T genome contains a substitution of 0.8–1.5 kb in the *env* region that represents the transforming gene, identified as v-*rel* (44, 50, 269). The v-*rel* is not present in nondefective REVs or other avian or mammalian retroviruses. Related sequences (c-*rel*) are present in the DNA of normal avian cells, including turkey cells from whence the oncogene was most likely transduced (44, 254). No endogenous REV sequences in host DNA have been recognized. The long terminal repeats (LTRs), 569 base-pairs in length (213) are efficient promoters in a variety of cell types (193). The complete genome sequence of two field isolates of REV from China and the United States has recently been published (GenBank accessions NC006934 and DQ387450, respectively).

Oncogene

The v-*rel* oncogene is transcribed in strain T-transformed lymphoid cells and produces a phosphoprotein product identified as $pp59^{v\text{-}rel}$. The v-*rel* protein is a member of the rel/dorsal family of proteins, which are related to nuclear factor kappa B and function as DNA-binding transcription factors (25, 198). It differs from the c-*rel* protein both in structure and transforming ability (see (95) and, unlike most other oncogene products, can be detected in both the cytoplasm and nucleus of transformed cells (see (26). The v-*rel* protein is usually complexed with cellular proteins (125, 136, 222). This protein is responsible for the acute oncogenicity of replication-defective strain T (26).

In several cases, REV isolates other than strain T have induced neoplastic disease within very short latent periods (70, 71, 185, 186). Examination of such strains for viral oncogenes of cellular origin may be of interest.

Proteins

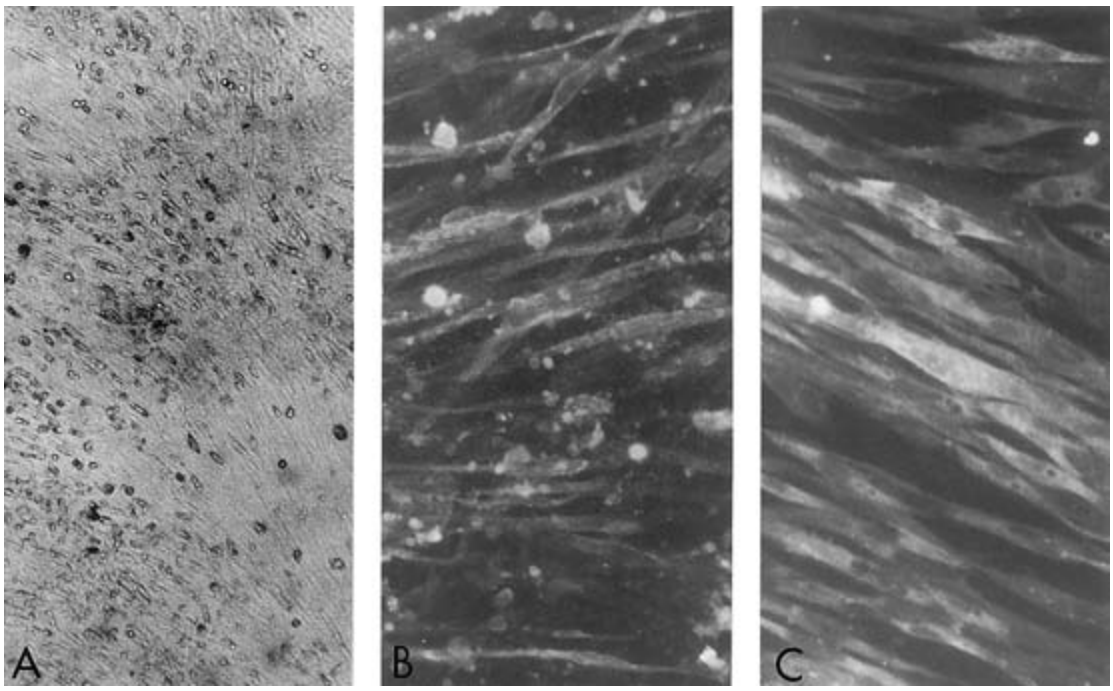
Like other retroviruses, REV genes express structural proteins, an envelope protein and a polymerase. The protein encoded by the v-*rel* gene is described above. The RNA-directed DNA polymerase (reverse transcriptase) differs structurally and immunologically from the comparable enzyme of leukosis/sarcoma viruses (19, 152). The preference of the REV polymerase for Mn^{2+} ions is a characteristic by which it can be differentiated from enzymes of other avian retroviruses (152, 205, 272).

A variety of polypeptides have been isolated from REV. The envelope protein is composed of two peptides, the gp90 surface unit (SU) and the gp20 transmembrane unit (TU) (239, 241). There are five *gag* gene-encoded structural proteins, p12, pp18, pp20, p30, and p10 (240). The C-terminal epitope of gp90 is located on the surface of infected cells (241). The gp90 protein is considered the immunodominant protein of the virus (62). The receptor binding regions have been mapped and structural differences compared to other retroviruses were noted (143). The 30-kD (p30) protein constitutes the major group-specific antigen that plays a role in viral particle assembly (252). Mosser *et al.* (157) located the two glycoproteins and two other proteins on the surface of the virions. Antiserum to p30 cross-reacted with p30 of several other REVs, thus establishing this protein as group specific (141).

Replication

Nondefective Strains

The cycle of replication *in vitro* is similar to that of other retroviruses and has been reviewed by Dornburg (68). Virus entry involves binding of the envelope glycoprotein to a specific receptor on the cell surface that has not yet been identified. Receptor



15.50. Acute (cytopathic) and chronic (noncytopathic) infection of chicken embryo fibroblasts inoculated with nondefective REV, strain T. A. Mild cytopathic changes 13 days after infection. Unstained, ×55. B. Cytopathic changes and viral antigens 13 days after infection demonstrated by indirect immunofluorescent staining. C. Chronically infected cultures 48 days after infection showing relatively normal-appearing cells, most of which contain cytoplasmic viral antigens as demonstrated by immunofluorescent staining. ×360.

binding results in interference with superinfection (85). Entry of virions into cells is probably accomplished by direct membrane fusion. Integration of the DNA provirus proceeds by different mechanisms in chicken and D17 cells. Viral RNA transcription and translation are initiated through promoter and enhancer sequences in the LTR. Two polyproteins are encoded, *gag-pol* and *env*; the *gag* precursor protein is myristylated. The encapsidation sequence is located in the *gag* gene. The final stage is budding of viral particles from the plasma membrane. Virus particle production was first noted at 24 hr (118), and maximum virus production occurred 2–4 days after infection in chicken cells (27, 88, 231).

Defective Strain

The replication-defective strain T virus requires a nondefective RE helper virus for replication (101). Oncogenicity of this strain is maintained during passage *in vivo* (195) or during culture of infected hematopoietic cells (101), but is rapidly lost during passage in chicken fibroblast cultures (234, 265) and dog thymus cells (1). Breitman *et al.* (29) showed this apparent attenuation in chick embryo fibroblast cultures to be due to the loss of the replication-defective, acutely oncogenic virus, which was completely absent after three passages; however, the nondefective helper REV continued to replicate.

Cytopathology

Replication of REV in avian fibroblast cultures is accompanied, in some but not all cases (234), by subtle cytopathic changes. Syncytial cell formation has been reported in infected cultures (51) but degenerative changes are more commonly seen (231). Temin *et al.* (232) proposed the following model: Infected cells synthesize unintegrated viral DNA, a part of which is integrated at multiple sites in the cellular genome. Progeny virus then superinfects the already infected cells, leading to an accumulation of unintegrated viral DNA. Cells with large amounts of unintegrated DNA die, while those cells able to prevent early superinfection have few copies of unintegrated viral DNA and survive.

The acute phase of cell killing (Fig. 15.50 A,B) lasts 2–10 days after infection and is followed by a state of chronic infection characterized by the disappearance of cytopathology and continued virus production (Fig. 15.50 C) (230, 231). This cytopathic effect is the basis of a plaque assay (36, 156, 231), but the method has not been widely used, perhaps because the cytopathology is somewhat inconsistent. Cho (46, 47) described a plaque assay in the QT35 line of chemically transformed Japanese quail fibroblasts.

Host Range

Cultured avian cells from many species, but especially chicken embryo fibroblasts, are susceptible to infection. However, certain mammalian cells support at least limited viral replication. Nondefective REV has been grown in D17 dog sarcoma cells (14, 250), Cf2th dog thymus cells (1, 214), normal rat kidney cells (122), mink lung cells (1), and bovine cells (13). D17 cells are susceptible and constitute a useful host system for virus propagation (250, 251), although REVs require some adaptation be-

fore high titers are obtained. Rat and mouse cells were only semipermissive for replication of REV, with blocks at different replication steps (73, 74). Chimeric vector particles containing the REV-A matrix protein infected mammalian cells more efficiently than those containing the matrix protein of spleen necrosis virus strain (43). A wide range of avian species support REV replication *in vivo* but there is little evidence for *in vivo* replication of REV in nonavian species. Johnson *et al.* (116) reported the presence of antibodies in humans, monkeys, horses and goats but this report requires confirmation. Although Koo *et al.* (126) reported that REV-A-based vectors could infect human cells, recent evidence indicates that REVs do not infected human cells due to the inability to bind to a cell surface receptor (93). Further, some batches of a commonly used D17.2G packaging cell line appear to be contaminated with murine retroviruses that produce, when infected with REV, pseudotyped virions that can infect human cells (93).

Pseudotypes

The envelope component of nondefective REV forms pseudotypes with Rous sarcoma virus (201, 242) and with vesicular stomatitis virus (119). The pseudotype virus can be neutralized by antiserum to REV; Crittenden *et al.* (55) has used this principle to detect REV antibodies in test sera.

Insertional Mutagenesis

Like other retroviruses, replication of REV requires integration of proviral DNA into the host cell genome. However, REV proviral DNA also can integrate into the genomes of large DNA viruses including Marek's disease virus (110) and fowl poxvirus (100). Insertions apparently result from co-infections of REV and a recipient DNA virus, and occur both *in vitro* and *in vivo* (59). Most insertions consist of a solitary LTR, sometimes with partial deletions, (117, 155). However, full-length, infectious REV genomes have been detected in turkey herpesvirus (111). In fowl poxvirus, a nearly full-length, infectious REV provirus has been detected in certain strains (90, 100, 124, 215). Such proviral insertions have been detected in fowl poxvirus stocks lyophilized for over 50 years (124). This phenomenon could be important because of the potential for REV insertions to change the biological properties of the recipient organism and because infectious clones of REV packaged in other viruses may provide a novel and important mechanism for transfer of infection (see section on horizontal transmission).

Strain Classification

The different isolates of REV are remarkably uniform in antigenicity (32, 183, 265) and, except for defective strain T, have similar structural and chemical properties (19, 120). Although REVs belong to a single serotype (45), three subtypes were identified on the basis of neutralization tests and differential reactivity with monoclonal antibodies (45, 56). Viruses of subtype 1 and 2 could not be differentiated by receptor interference (85), thus confirming the absence of major differences between subtypes. REV isolates differ also in certain biologic properties, including pathogenicity (184) and replication *in vivo* (2), but such differences have not been the basis for strain classification.

Laboratory Host Systems

Cell Cultures

Fibroblasts from several avian species and certain cell lines, such as QT35 quail sarcoma cells (47, 53) and D17 dog osteosarcoma cells (14, 250), are susceptible to infection with nondefective REVs. In infected cultures, antigens (Fig. 15.50B,C), virus particles, proviral DNA, cytopathology, and reverse transcriptase may be detected and serve as criteria for virus assay. When the cultures are grown under agar, foci of cells containing immunofluorescent antigens can be localized and used as the basis for a quantitative fluorescent focus assay (183). Duck embryo fibroblasts may be preferred for demonstration of cytopathic effects (12). However, chicken bone marrow-derived macrophages appear resistant to infection (33).

Embryos and Birds

Other laboratory host systems for REV include chicken embryos (2, 211) and a variety of avian species including young chickens, Japanese quail, ducks, geese, turkeys, pheasants, and guinea fowl (15, 185, 234). Embryos and animals may respond to infection by development of specific lesions, viremia, or antibodies.

Cell Lines

Hematopoietic cells transformed *in vivo* or *in vitro* by the replication-defective REV have been developed into continuous cell lines; the cell types and surface markers vary based on the strain of helper virus and whether transformation occurred *in vivo* or *in vitro* (see 26, 105). A line of transformed chicken embryo fibroblasts has also been developed (87). Nonproducer clones can be isolated that produce pseudotypes when infected with nondefective REV strains (102). The cells possess surface viral antigens that co-cap with antigens of the major histocompatibility complex (140). Cell lines have also been derived from chronic lymphomas induced by nondefective strains of REV (169, 186). Cell lines induced by *in vitro* transformation of spleen cells with defective REV can serve as useful expression systems for transfected foreign genes (182, 204) or as substrates for the propagation of other viruses (188). Some of these transformed cell lines elaborate growth factors or cytokines (67, 91, 94).

Pathobiology and Epidemiology

Incidence and Distribution

REV infection is common, but not ubiquitous, in flocks of turkeys, ducks, chickens and certain other avian species throughout the world. The prevalence of seropositive flocks and the proportion of seropositive birds in an infected flock both increase with the age of the flock. Aulisio and Shelokov (6) found nearly half of 92 chicken flocks in the United States were seropositive. Subsequent studies, reviewed by Bagust (12), have established the presence of REV antibodies (or virus) in a number of countries. Infection appears to have been endemic in Japan as early as 1964 (244, 270). Recent surveys showed antibodies in 34–75% of chicken flocks in Korea (209) and Egypt (3). Seropositive chicken flocks are still common in the United States (Witter, unpublished).

In contrast, the incidence of REV-associated clinical disease in commercial poultry varies from sporadic to negligible. Acute reticulum cell neoplasia is not seen in the field. The runting disease syndrome has been primarily seen following vaccination of young chickens with REV-contaminated vaccines (113, 121, 273), although REV has been demonstrated in flocks with a naturally-occurring immunodepressive disease in Korea (209). In vaccine-induced outbreaks, a high proportion of chickens are typically affected.

Chronic neoplasia associated with REV infection is also rare. Field cases of lymphomas in turkeys have been described in the United States (54, 176, 221, 261, 266), England (145), and Israel (107). Losses from mortality and condemnation at slaughter in affected flocks could be as high as 16–20% (145, 176). Lymphomas associated with REV infection have also been reported in wild turkeys (99, 134). REV-associated lymphomas in chickens have also been reported (60, 107, 149, 173, 174) but are rare. Chronic neoplasia associated with REV has also been occasionally observed in ducks (97, 177, 179), quail (39, 203, 243), pheasants (71), geese (70), peafowl (151) and prairie chickens (72, 78, 276).

Administration of REV-contaminated vaccines to chickens may also induce chronic lymphomas (12, 83, 187).

Natural and Experimental Hosts

Natural hosts for REV infection include turkeys, chickens, ducks, geese, pheasants, Japanese quail, peafowl, and prairie chickens. Experimental hosts include all of the above species, as well as guinea fowl. Chickens and turkeys have been most frequently employed as experimental hosts.

Transmission, Vectors, and Carriers

Horizontal Transmission

Experimentally, REV can be transmitted by direct physical contact with infected chickens, turkeys and ducks (132, 160, 175). Horizontal transmission may be influenced by the host species (183) and the virus strain (262, 274), and was not detected when chickens were separated by wire mesh (10).

In nature, many flocks develop REV infection at older ages (262) where direct contact with infected birds can be excluded. Environmental sources of infection may include contaminated poultry houses, insects and other biological reservoirs. Poultry house environments can become contaminated with virus shed by infected birds since REV has been detected in feces and cloacal swabs (8, 180, 268, 274), other body fluids (10), and litter (249). Virus present in poultry house environments, if transported on fomites or insufficiently disinfected, could potentially serve as a source of infection for other flocks. However, this does not seem to explain the high rates of flock infection because, in flocks infected at later ages, virus is difficult to isolate (262) and the quantity of shed virus is probably limited. Furthermore, REVs, like all retroviruses, are quickly degraded outside the host at ambient temperatures (38).

Insect transmission is another possible source of horizontal transmission of REV. Virus could be recovered for brief periods in *Triatoma infestans* and *Ornithodoros moubata* after feeding on infected chickens (235, 236) but attempts to propagate REV in cultures of *Aedes albopictus* were unsuccessful (190). Motha *et al.* (166) isolated virus from 7 of 39 batches of mosquitoes (*Culex annulirostris*) in contact with viremic chickens and demonstrated apparent mechanical transmission of the infection to recipient chickens exposed to infected mosquitoes. Davidson and Malkinson (62) found that mosquitoes fed on infected blood for 96 hr retained infectious virus for an additional 5 hr but no transmission studies were conducted. Mosquito transmission has been proposed to explain why flocks seroconvert more commonly during summer months (61, 166) and why there is a high prevalence of infection in Southern states (262, 264). However, if insects are to be an important mode of infection for older poultry flocks, an ample reservoir of infected donor animals would be required, but the proportion of viremic chickens in field flocks at any given time appears too low to constitute the necessary biological reservoir. Novel viral reservoirs such as fowl poxvirus that may contain infectious clones of REV (90, 100, 215) and is also transmitted by mosquitoes could also be considered. Thus, mechanisms for horizontal transmission among commercial flocks and the identity of biological reservoirs of infection are poorly understood and further study is needed.

Vertical Transmission

Chickens, turkeys and ducks with persistent viremias may transmit infectious REV to progeny, although usually at low frequency (compared to avian leukosis virus). McDougall *et al.* (147) isolated virus from 2 of 25 embryos from tolerantly infected turkey hens. Similar low rates of viral shedding and transmission were documented for tolerantly infected chickens (8, 10, 245, 268), although Motha and Egerton (165) reported transmission to over 50% of chicks in an experiment in which eggs were incubated within 24 hr of lay. Albumen samples from tolerantly infected hens frequently contained RE viral gs antigen, although at low levels; infectious virus was rarely isolated (268). Vertical transmission may occur at higher rates in ducks, since virus was isolated from 87% of embryos derived from tolerantly infected females in one trial (160). Vertical transmission from nontolerantly infected chickens is even less common, but one exceptional antibody-positive, virus-positive turkey hen transmitted virus to 6 of 21 progeny (266).

Although semen from tolerantly infected turkeys contains infectious virus (148, 266), the role of the tom in vertical transmission is not clear. McDougall *et al.* (147) found that previously nonexposed turkey hens inseminated with infected semen produced infected progeny, but, in contrast, Witter and Salter (266) found the frequency of vertical transmission was no greater from hens mated with viremic males than with hens mated with nonviremic males. Furthermore, they found no evidence in turkey breeders or congenitally infected progeny of clonal insertions of proviral DNA that would be indicative of genetic transmission (266). Male transmission has received less attention in chickens, but Salter *et al.* (199) found RE proviral DNA in 10 of 820 chicks from matings of viremic males and nonviremic females. Clearly, a role for the male in vertical transmission of REV has not been excluded and needs further study.

The possibility that congenitally transmitted virus could be transferred by the sequential use of needles during the administration of Marek's disease vaccines to embryos or newly hatched chickens should also be considered (12).

Contaminated Biological Materials

Accidental contamination of virus and vaccine stocks with REV has been observed on a variety of occasions. The use of REV-contaminated fowl pox (23, 83) or Marek's disease (113, 274) vaccines has been documented, sometimes resulting in major economic consequences. Certain stocks of avian myeloblastosis virus, for many years distributed as a source of reverse transcriptase for biochemical purposes, contained a low level of REV (264). Quality control procedures to exclude REV from licensed poultry biologics are not uniformly effective (75). Standard assays may not detect REV contamination in vaccines such as fowl pox (75, 80). REV continues to be found in some (65, 90, 100, 215), but not all (155), fowl pox virus vaccines. REV has also been detected in stocks of *Plasmodium lophurae*, which was serially passed in ducks (138, 238). The occurrence of such problems points to further mechanism for increasing the distribution of this virus in nature.

Incubation Period

The runting disease syndrome includes a number of nonneoplastic disease processes that are not coordinately expressed and vary depending on virus strain and several other factors. Atrophic changes in the bursa and thymus can be seen as early as 3 days after infection (167). Weight depression in infected chicks can be detected as early as 6 days of age (158) and persists for life. By the second week postinoculation, chickens developed microscopic nerve lesions (265) and had depressed immune responses (263).

Chronic neoplastic responses occur after moderate or long incubation periods. Chickens developed bursal-derived B-cell lymphomas 17–43 wk after inoculation (268). Chronic nonbursal lymphomas with latent periods as short as 6 wk have been described in line 6_3 and line 0 chickens following experimental infection with the spleen necrosis or chick syncytial strains of nondefective REV (267). REV-associated lymphomas in turkeys occurred between 15 and 20 wk of age (145, 176). In transmission studies, lymphomas were induced after 8–11 wk (175) or 11–12 wk (145). Chronic lymphomas occur between 20 and 30 wk in the domestic goose (70), at 4–24 wk in ducks (97, 177, 179). Experimental inoculation of newly hatched ducks induced lymphomas and other neoplasms between 8 and 30 wk (135, 160).

For acute reticulum cell neoplasia, the incubation period can be as short as 3 days, but death occurs more commonly 6–21 days after inoculation (210). Because of the short latent period and high mortality, Bose has referred to defective REV strain T as the most virulent of all retroviruses (26).

Clinical Signs

Chickens with runting disease syndrome may be notably stunted and pale (167). Weights of infected chickens may be 20–50% lower than controls by 3–5 wk after infection (263, 265). Weight depression has also been seen in infected ducks (183). Some chickens may have abnormal feather development, termed *Nakanuke*, i.e., wing feathers with adhesion of the barbs to a localized section of the shaft (131). Lameness or paralysis is rare even in birds with gross nerve lesions. Mortality is rare in chickens (263) but affected birds in commercial flocks are usually culled prior to death; a culling loss of over 50% between 5 and 8 wk was described in one flock (227).

Birds developing chronic lymphomas become depressed prior to death but show few specific clinical signs. Similarly, newly hatched chickens or turkeys that develop acute reticulum cell neoplasia following inoculation with defective strain T show few clinical signs due to the rapid onset of the disease, and mortality rates often reach 100% (211, 234).

Pathology

Runting Disease Syndrome

In chickens, the principal lesions include runting (167, 265), atrophy of the thymus and bursa of Fabricius (167), enlarged peripheral nerves (265), abnormal feather development (129, 130), abnormal proventriculitis (113), enteritis (145), anemia (121, 138), and necrosis of the liver and spleen (184, 238). These are often accompanied by depression of cellular and humoral immune responses (32, 40, 107, 121, 263). The acute hemorrhagic or chronic ulcerative proventriculitis observed in field cases (113) could not be reproduced by Bagust *et al.* (9) with a similar isolate.

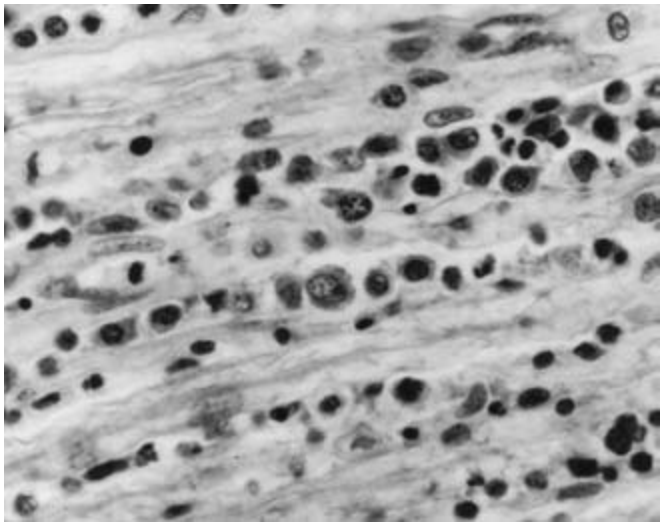
It is unclear whether the proliferative lesions in enlarged peripheral nerves are neoplastic or inflammatory; however, nerve lesions often occur in the absence of other neoplasms (265). Grossly, nerves are only modestly enlarged—up to 2× the diameter of controls. The infiltrating cells, which include lymphocytes and plasma cells, are shown in Figure 15.51.

At least parts of the runting disease syndrome occurs in ducks inoculated with the spleen necrosis or duck infectious anemia strains of REV (138, 238). Hematocrit values in ducks inoculated with spleen necrosis strain can be as low as 20%, compared to 35% for control ducks (238). Enlarged nerves (175, 176) or enteritis (145) have been observed in turkeys with RE-related chronic lymphomas.

Genetic differences in susceptibility have not yet been described; chicks from lines of different susceptibility to Marek's disease were equally susceptible to the development of nerve lesions following inoculation with REV (265). Most nondefective REV strains, when inoculated at hatch, induce high frequencies of gross lesions (183, 263) but others, such as chick syncytial strain, may induce few, if any, lesions (263).

Chicken Bursal Lymphoma

Chickens inoculated with the nondefective chick syncytial or T strains developed B-cell lymphomas, involving principally the liver and bursa of Fabricius (258, 268). The gross lesions were nodular or diffuse lymphoid lesions in the liver and other visceral organs, including nodular lesions in the bursa of Fabricius, which were indistinguishable from lymphoid leukosis (Fig. 15.52). A few birds may develop sarcomas or adenocarcinomas. The frequency of lymphomas was influenced by virus strain and whether a tolerant infection had been induced (268). Interestingly, coin-



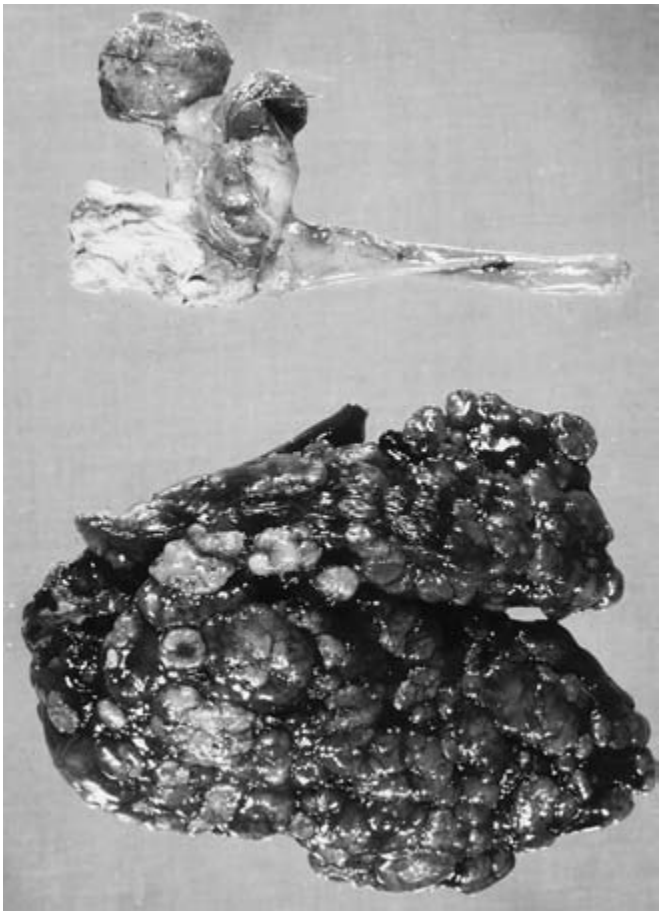
15.51. Microscopic lesions in a peripheral nerve of a chicken inoculated with nondefective strain T REV. Infiltrating cells consist of mature and immature lymphocytes and plasma cells.

fection of chickens with serotype 2 Marek's disease virus enhanced the incidence of REV bursal lymphomas (5) as had also been reported for lymphoid leukosis (7).

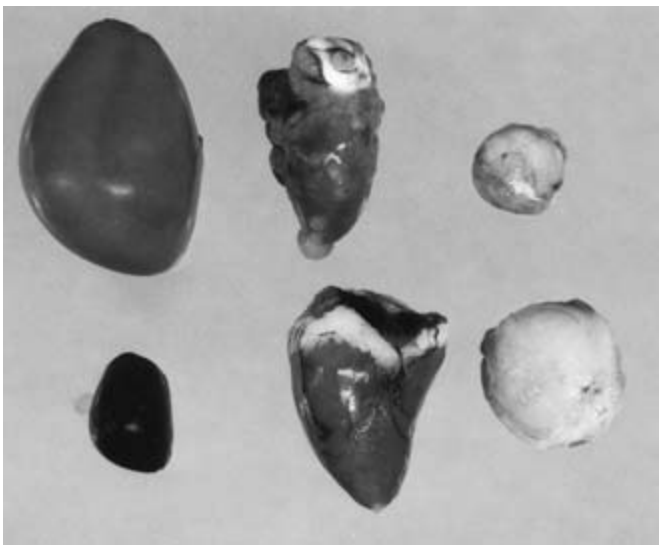
The tumor cells, which appeared histologically as uniform populations of lymphoblasts, were identified as B cells by IgM and other B-cell specific markers (169, 268). The bursal dependency of this tumor was confirmed by the finding that chemically or surgically bursectomized chickens were refractory to tumor development (82). Bursal lymphomas may not always be present in field cases. Grimes *et al.* (96) observed what may be similar lymphomas in two chickens at 22 and 24 wk after inoculation with a field strain of REV, but no bursal involvement was reported. However, typical bursal lymphomas were observed in two chicken flocks following administration of a REV-contaminated fowl pox vaccine (83).

Chicken Nonbursal Lymphoma

Chronic nonbursal lymphomas have been described in certain lines of chickens following experimental infection with the spleen necrosis or chick syncytial strains of nondefective REV (267). Grossly, these lymphomas appear as focal or diffuse lymphoid infiltrations, usually producing enlargements of the thymus, liver, and spleen or focal lesions of the myocardium (see Fig. 15.53). The bursa of Fabricius is not involved. Nerve enlargements, probably from concomitant expression of the runting disease syndrome, may be seen. Histologically, the tumors appear to be a uniform, immature lymphoreticular cell that lacked B-cell markers and did not express MATSA, a cellular antigen associated with Marek's disease tumors (267) and also expressed on activated T lymphocytes (144). The principal tumor cell type is a CD8+ T cell but Ia antigens are not expressed (52). Only certain chicken lines, especially line 6_3, are susceptible (267). Thusfar, T-cell lymphomas have not been documented in the field.



15.52. Bursal lymphoma in a chicken. Note gross lymphomas in the liver and bursa of a chicken 25 weeks after inoculation with the nondefective chick syncytial strain of REV.



15.53. Nonbursal lymphoma in a chicken 48 days postinoculation with the nondefective spleen necrosis strain of REV. Note enlargement of spleen, nodular lymphomas on heart, and bursal atrophy of infected chicken (top row). Organs from age-matched control chicken are shown in the bottom row. (267).

Turkey Lymphoma

Chronic lymphomas in turkeys are characterized by gross lymphoid infiltrations in the liver, intestine, spleen, and other visceral organs. Paul *et al.* (175) and McDougall *et al.* (145) both described lymphomatous lesions in the bursa, but this lesion was not common. Grossly, livers were enlarged up to 3–4× normal size. Some spleens were enlarged while others showed little enlargment but focal lesions were present. Intestines were thickened and some showed annular lesions. Histologically, the lesions were composed of uniform populations of lymphoreticular cells (145). Crespo *et al.* (54) described T-cell lymphomas associated with an outbreak of reticuloendotheliosis in turkeys.

Lymphomas of Other Species

Various other species develop chronic lymphomatous lesions associated with REV infections. Gross lesions are not greatly different for those described for chickens and turkeys. Lesions reported in ducks include enlarged livers and spleens with diffuse or focal involvement, intestinal lesions, and infiltrations in skeletal muscle, pancreas, kidneys, heart and other tissues (97, 135, 160). Perk *et al.* (179) described an outbreak in ducks characterized by generalized leukemia in addition to visceral lymphomas. Similar tumors were described in geese, and included also occasional lymphoproliferative lesions in the bursa of Fabricius (70). Outbreaks in both pheasants and prairie chickens were characterized by cutaneous lesions on the head and mouth in addition to nodular lymphomas in visceral organs (71, 72, 276). Outbreaks in quail, attributed to REV, were characterized by liver and spleen enlargements (203) or intestinal lesions (39). Histologically, tumors from all these species generally resembled those described for chickens and turkeys; the identify of the target cell has not been determined.

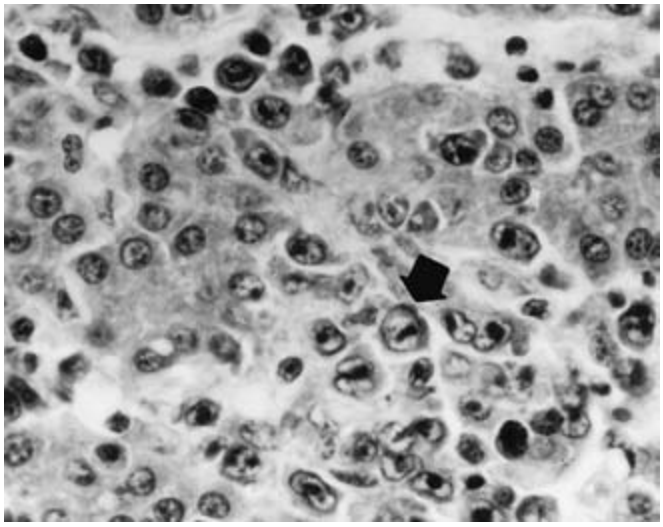
Acute Reticulum Cell Neoplasia

The pathology of the acute reticulum cell neoplasia has been well described (195, 211, 234). The affected birds develop large livers and spleens with infiltrative focal or diffuse lesions. Lesions are also common in the pancreas, gonads, heart, and kidney. The blood shows a decrease in heterophils and an increase in lymphocytes (228), leading to a frank leukemia a few hours before death (212). The serum transferrin level is elevated (237) and Shen (212) reported elevated globulin and decreased albumin concentrations.

Histologic changes are generally characterized by the infiltration and proliferation of large vesicular cells, variously described as mononuclear cells of the reticuloendothelial system (234) or primitive mesenchymal cells (195, 211). Some lesions are composed almost solely of such cells, whereas others include also a moderate to heavy population of smaller lymphoid elements, probably indicating a host immunologic response to the primary lesion. Areas of necrosis in association with the neoplastic lesions are also frequent. A typical liver lesion is shown in Figure 15.54.

Multiple Syndromes

Lesions of the different types can be observed in the same experiment, or even in the same bird. Nondefective REV strains may first induce lesions of the runting disease syndrome and lymphomas may occur later in the survivors, sometimes accompanied by nerve enlargements. Chickens inoculated with replication-defective, acutely transforming strain T, especially those surviving the acute disease, may develop lesions associated with the nondefective strain T helper virus.



15.54. Microscopic lesions of acute reticulum cell neoplasia (reticuloendotheliosis) in the liver of a chicken inoculated with replication-defective, acutely transforming strain T REV. The liver is infiltrated with large primitive reticular cells (arrow).

Pathogenesis

Virus Infection

Once REV infection is established in a susceptible host animal, it proceeds in one of two paths, which to a large extent determine pathologic outcomes.

Tolerant infection, i.e., persistent viremia in the absence of antibody, is induced readily in chickens by embryo inoculation (107, 268) and by vertical transmission of virus from infected dams (10). Persistent infections occur rarely following inoculation at hatching (8, 147, 268) depending on the strain of chicken (81); and are unlikely to occur if exposure occurs at later ages. Persistent infections also occur in turkeys (147, 266). Some birds with persistent infections develop antibody responses. Tolerant infection is associated with higher rates of vertical transmission and tumor development, and birds are typically stunted and immunodepressed.

In birds exposed at hatching or later, however, a transient viremia followed by the development of antibodies is the most common consequence of infection (8, 263). Bagust and Grimes (8) described the persistence of noninfectious RE viral antigens in the blood for several weeks following the disappearance of infectious virus. Transient infection only rarely results in vertical transmission, immunosuppression, or tumor development. Infection of older birds rarely results in clinical disease (180, 183, 262, 274) except, perhaps, in turkeys in which lymphomas have been observed following contact exposure (147, 148, 175).

Various other factors influence susceptibility of avian hosts to infection or disease. No genetic cellular resistance has been recognized. However, some differences in the pathologic response of lines or families has been recognized in chickens (81, 207, 212, 267) and quail (233). However, differences were not apparent when two chicken lines were challenged with serial dilutions of the replication-defective strain T (210). Although endogenous leukosis virus genes had no influence on tumor induction or antibody response following exposure of chickens to REV, virus was isolated more frequently from chickens with *ev*2 than from chickens lacking this gene (55). A cellular resistance (interference) due to viral envelope gene expression has been described in cultured D17 cells (64) (85). Maternal antibodies appear to limit susceptibility to infection (218).

Runting Disease Syndrome

The pathogenesis of the various lesions associated with the runting disease syndrome has not been elucidated. Stunted chickens did not consume less food, but had marked reduction of phosphoenolpyruvate carboxykinase, a key gluconeogenic enzyme in the liver (92). The adherence of feather barbules to the shaft (Nakanuke) is apparently due to REV-induced necrosis of feather-forming cells early after inoculation (225). Mussman and Twiehaus considered the microscopic lesions of chicks with runting syndrome to resemble a graft-versus-host reaction (167), but a specific autoimmune component has not been identified.

Chronic Lymphomas

Noori-Daloii *et al.* (172) found in REV-induced bursal lymphomas that the DNA proviral genome of REV was integrated adjacent to c-*myc*, a cellular oncogene important in the induction of lymphoid leukosis by avian leukosis virus. The molecular mechanism by which c-*myc* is activated by insertion of REV proviral DNA has been described (89, 194, 224). The proviral insert often contains major deletions that prevent the expression of infectious virus (223). Based on pathology, proviral insertional activation of c-*myc*, and enhancement by serotype 2 Marek's disease virus, bursal lymphomas induced by REV and avian leukosis virus appear indistinguishable—a rare case in which the same disease is caused by two unrelated viruses. However, some subtle differences have been noted. For example, chickens of lines resistant and susceptible to lymphoid leukosis were uniformly less susceptible to lymphoma induction by REV than by avian leukosis virus (81), and the REV lymphomas usually require longer latent periods than those induced by avian leukosis virus.

For nonbursal lymphomas, the molecular mechanism of oncogenesis also involves insertional activation of c-*myc*, but differs from that in bursal lymphomas; the strong tendency for the provirus to be oriented in the same direction as c-*myc* in bursal lymphomas was not observed in nonbursal lymphomas (112).

Critical comparisons between chronic lymphomas in turkeys and chickens have not been made and there is no evidence that a common mechanism of oncogenesis exists. In ducks, the frequency of experimentally induced REV lymphomas was not affected by embryonal bursectomy (135), indicating that these tumors may not be of B-cell origin.

Acute Reticulum Cell Neoplasia

The target cell transformed *in vivo* by replication-defective strain T (with REV-A helper virus) expresses T lymphoid and myeloid markers (16). These cells also express surface MHC class I and II antigens, as well as interleukin-2 receptor (103), and are immunoglobulin M (IgM) negative but vary in expression of CT3 (16). Similar tumors were induced in chemically bursectomized chickens (16). Inoculation directly into the thymus induced thymomas composed of T and B cells (26). On the other hand, defective strain T, when associated with chick syncytial virus helper virus instead of REV-A, induces IgM-positive B-cell lymphomas with rearrangements of the heavy- and light-chain immunoglobulin loci (17, 18). Thus, cell tropism appears to be determined, in part, by differential effects of the helper viruses on lymphoid populations.

Neoplastic transformation in acute reticulum cell neoplasia is mediated by the oncogene, v-*rel*, contained within the replication-defective strain T virus. Transformation does not require the presence of a helper virus (133). Lymphoid cells transformed by strain T *in vitro*, but which produce no infectious virus, will produce typical RE when transplanted into syngeneic recipients (133, 197).

Immunity

Humoral Responses

Birds with nontolerant infections develop robust antibody responses. Antibodies have been detected as early as 16–21 days after inoculation in chickens (32, 161), but 6–10 wk may be required in contact-exposed birds (107, 132, 147). Antibody titers may decline with age (8, 32, 268), but McDougall *et al.* (147) detected neutralizing antibodies at high frequency in experimentally infected turkeys through 40 wk. Most birds that develop tolerant infections do not develop humoral immune responses, although a few tolerantly infected chickens ultimately develop antibodies (158). The presence of antibodies may influence tumor susceptibility as chemically bursectomized quail were more susceptible than controls to inoculation with a field isolate (185).

Cellular Responses

Major histocompatibility complex (MHC)-restricted cytotoxicity against lymphoblastoid cell lines transformed with defective REV has been described in chickens within 7 days after inoculation with defective or nondefective RE viral strains (139, 253). This response appears to be mediated by activated (MHC class II+) CD8+ T cells (127). However, NK cells were not activated (202). The induction of cytotoxic T cells by REV has been used as a general indicator of immune response in the study of other avian viruses (188).

Immunodepression

Humoral and cellular immune responses are frequently depressed in chickens infected with nondefective REV strains. Depressed antibody responses to Marek's disease virus and turkey herpesvirus (32, 121), Newcastle disease virus (107, 273), as well as to sheep erythrocytes and *Brucella abortus* (263) are documented. The magnitude of antibody depression is influenced by

the dose and strain of virus, and primary responses are more severely affected than secondary (263). Barth and Humphries (17) found that different strains of nondefective REV varied in ability to induce bursal atrophy and in suppression of B-cell populations available for transformation by v-*rel*. Studies on chimeric viruses derived from REV-A and chick syncytial virus showed that regions in both *gag* and *env* genes were associated with the strong immunodepressive ability of REV-A (86).

Spleen cells from chickens infected with replication-defective strain T were suppressed in their ability to respond to the mitogen, phytohemagglutinin (40, 206). This effect is associated with the nondefective helper virus in strain T stocks (42) and is mediated through a population of suppressor cells (41, 196). The suppressor cells could be demonstrated only through the third week after infection (197). Other cellular immune responses inhibited by REV infection include mixed lymphocyte reaction and allograft rejection (248).

Witter *et al.* (263, 268) found depression of humoral responses and mitogen responsiveness was transient following infection with the chick syncytial strain, but persisted through 10–19 wk in chickens tolerantly infected with nondefective strain T. Infected chickens were more susceptible to the development of a Marek's disease tumor transplant (35), to reactions from infectious laryngotracheitis vaccine (161, 217), to natural fowl pox virus infection (164), to infectious bronchitis virus (217), and to mortality induced by *Eimeria tenella* (163) and *Salmonella typhimurium* (162). No increase in susceptibility to Marek's disease virus was noted (31), but Witter *et al.* (263) demonstrated interference by REV infection with immunity induced by turkey herpesvirus against Marek's disease in chickens. Humoral immunodepression was also seen in ducks infected with a field isolate of REV (135). In the field, immunodepression is probably the most important consequence of embryo- or vaccine-derived REV infections but is less likely to result from contact infection (262) and has not commonly been associated with seropositive flocks. REV-induced immunosuppression has recently been reviewed by Zavala (275).

Tumor Immunity

A protective immune response against the acute neoplasia induced by strain T has been described. Regression of strain T-induced wing-web tumors was partially abrogated by bursectomy, thymectomy, and bursectomy-thymectomy (137). Serum from hyperimmunized chickens was protective against tumor development even after absorption to remove antiviral antibodies (104), thus suggesting the existence of tumor-specific transplantation antigens on RE tumor cells. Chickens immunized with purified or inactivated preparations of nondefective strain T helper virus were resistant to challenge with acutely transforming strain T preparations (21). However, immunization with empty virions (150) did not provide protection.

Diagnosis

A diagnosis of RE requires not only the presence of typical gross and microscopic lesions, but also the demonstration of REV. Because REV, unlike avian leukosis and Marek's disease viruses, is not yet as ubiquitous, the demonstration of infectious virus, viral antigens, and proviral DNA in tumor cells has diagnostic value. Diagnostic techniques have been reviewed by Fadly *et al.* (84).

Isolation and Identification

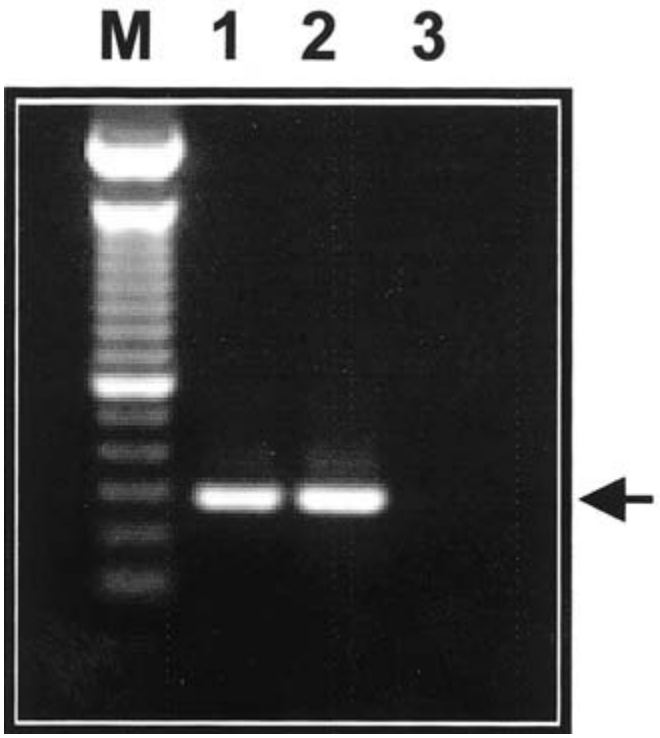
Viremia with REV is typically low titered and transient, except following congenital transmission or embryo inoculation that leads to tolerant infection. Birds with lesions are normally a good source of virus. Virus may be isolated by inoculation of susceptible tissue cultures with tissue suspensions, whole blood, plasma, or other inocula. In general, cellular inocula are preferred over cell-free inocula, since the former usually contain higher titers of virus than the latter. Because cytopathic effects in cell cultures may not be seen on primary isolation, cultures should be maintained through at least two blind 7-day passages. The presence of REV is confirmed by the demonstration of viral antigen in infected cell culture using polyclonal or monoclonal antibodies (56), by immunofluorescence (265), immunoperoxidase staining (36), complement fixation (219), or enzyme immunoassay (57, 109). In comparative studies, enzyme immunoassays were more sensitive than complement fixation tests (57) and indirect immunofluorescence was more sensitive than indirect immunoperoxidase or immunoelectron microscopy (171). A convenient and sensitive indirect immunofluorescent assay conducted in 96-well plates (45) has been used for virus isolation from field samples (266).

Virus isolated by this procedure may be identified by reproduction of the typical disease in experimental animals and by further neutralization tests. Virus isolates may be assigned to antigenic subtypes by the differential reactivity of monoclonal antibodies in immunofluorescent assays (45). Subtyping of isolates may have value for epidemiologic studies.

Detection of proviral DNA by polymerase chain reaction (PCR) assays that amplifies the 291 base pairs product of REV LTR (Fig. 15.55) has been shown to be a sensitive and specific method for detection of various strains of REV in infected chicken embryo fibroblasts as well as in blood and tumors of infected chickens (4, 209). This procedure appears useful for tumor diagnosis (58, 60, 61, 63) and the evaluation of vaccines for possible REV contamination (80, 83, 100, 191, 226, 229). Garcia *et al.* (90) found that assays amplifying REV envelope and REV 3′ LTR sequences provided a more accurate assessment of REV provirus than PCR assays that amplify the REV 5′ LTR region. Although PCR assay can be used in lieu of antigen detection assays for demonstration of infection in inoculated cell cultures or directly in tissue samples it may not be as well suited as enzyme immunoassay for large-scale testing.

Serology

Confirmation of REV infection by serologic procedures involves the detection of antibodies in sera from chickens inoculated with suspect isolates or from affected chickens. Antibodies are induced with various frequencies and persist for varied periods. The most sensitive test for detection of antibodies to REV is



15.55. PCR analysis of DNA isolated from chicken lymphomas using primers specific for REV. Lanes: M, 100 bp ladder; 1, experimentally induced REV lymphoma (positive control); 2, unknown lymphoma (test sample); 3, uninfected chicken embryo fibroblasts. The expected 291 bp PCR product (arrow) is indicated. (Lupiani)

virus neutralization; briefly, samples of plasma or serum are reacted with REV and the neutralization of REV is determined by assay for residual virus using enzyme immunoassay or immunofluorescence. Immunoperoxidase plaque assay (36) has also been shown to be a sensitive and reliable method for detection of REV antibody. Specific antibody may be detected in the serum or egg yolk from exposed birds by indirect immunofluorescence (6, 265), virus neutralization (147, 183), agar gel precipitin (106, 159), enzyme immunoassay (34, 170, 220, 271) and pseudotype neutralization (55) tests. Enzyme immunoassay kits for antibody detection are commercially available. The agar gel precipitin test may detect viral antigen as well as antibody in sera (106, 107). Antibody tests are particularly useful in ascertaining the absence of viral exposure in specific-pathogen-free breeder flocks or flocks producing progeny for export.

Differential Diagnosis

The pathology of REV-induced tumors, particularly those of lymphoproliferative nature, can be confused with that of tumors seen in Marek's disease and lymphoid leukosis (76, 79, 246). Because avian tumor viruses are widespread and infection in the absence of tumor formation is common, in most cases virologic and serologic criteria rarely provide a definitive diagnosis. However, as previously stated, diagnoses of RE should be supported by virologic evidence of REV infection, as REV is not as ubiquitous as Marek's disease and avian leukosis viruses. Techniques based on immunocytochemistry with monoclonal antibodies to cellular, tumor and viral antigens, or molecular hybridization can be used in the differential diagnosis of avian viral lymphomas including RE.

Avian retroviral lymphomas in chickens originate from either B cells (RE, lymphoid leukosis) or T cells (RE), whereas Marek's disease lymphomas are of T-cell origin (see subchapters on Marek's disease and Leukosis/Sarcoma Group). The characteristics of target cells provide the basis for tests that distinguish among B- and T-cell lymphomas using monoclonal antibodies specific for cell surface antigens of B- and T-lymphocytes. Nondefective strains of REV have been shown to transform chicken B or T cells by integration within a cellular (c) *onc* gene such as c-*myc* resulting in the enhanced expression of such gene and thereby initiating the lymphomagenic process (112, 224). These molecular changes are the bases for testing tumor DNA for the definitive diagnosis of REV-induced lymphomas.

The PCR assays for RE (see "Isolation and Identification"), Marek's disease (see subchapter, Marek's Disease) and exogenous avian leukosis virus (see subchapter, Leukosis/Sarcoma Group) can be helpful in the differential diagnosis of RE. For instance, because Marek's disease lymphomas should contain a significant proportion of Marek's disease virus-infected cells, compared to latently infected tissues, Marek's disease lymphomas should have more infected cells, each with greater number of viral copies thus resulting in higher total estimates of viral load by PCR analysis. Quantitative PCR analysis may be conducted by at least two methods, a quantitative-competitive method (189) and real-time PCR. Nonquantitative PCR assays are probably of little value for diagnosis of Marek's disease because of the potential to detect MDV DNA in the absence of lymphomas. Also, PCR has been shown to detect REV-LTR sequences from lymphomas and brains of REV-infected chickens, but not from DNA from Marek's disease or lymphoid leukosis lymphomas (4). However, diagnosticians should always keep in mind that PCR assays demonstrate the presence or absence of the respective virus and thus posess the same limitations for tumor diagnosis as virus isolation.

Chronic neoplasia in the chicken where the tumors are of bursal origin cannot usually be differentiated from lymphoid leukosis on pathologic criteria (258); virologic, serologic, or PCR tests providing infection can be established for one virus and excluded for the other. In addition, RE or lymphoid leukosis tumors should contain proviral DNA sequences of the respective virus inserted near the c-*myc* gene, a characteristic that could permit differentiation of lymphomas by molecular hybridization. Using appropriate REV probes such as pSNV, or c-*myc probes*, Southern blots and hybridization analysis of tumor DNA is used to detect insertion of REV provirus and alteration in c-*myc*, respectively.

Chronic neoplasia in the chicken in which bursal tumors are lacking or in which the latent period is too short for that of lymphoid leukosis must be differentiated from Marek's disease. Here too, pathologic criteria are insufficient and virologic assays (including PCR) may be helpful. The pp38 antigen of Marek's dis-

ease virus, occasionally expressed in Marek's disease lymphomas (see subchapter, Marek's Disease), is not present in RE lymphomas. Also, MHC class II (Ia) antigens are reported to be present on Marek's disease lymphoma cells (202) but absent on RE nonbursal lymphoma cells (52). Recently, Witter *et al.* (260) introduced a diagnostic strategy for the differential diagnosis of virus-induced lymphomas in chickens.

The acute reticulum cell neoplasia syndrome is not known to occur in the field and is unlikely to require differential diagnosis when experimentally reproduced in the laboratory. A new syndrome of broiler chickens characterized by reticuloendothelial proliferation in the spleen and liver, and resulting in condemnation loss at processing, has been confused with RE (98, 247), but can be distinguished by the absence of RE antigens and proviral DNA in the lesions (256). Virus detection tests including PCR have been used to confirm the diagnosis of REV-induced orbital lymphosarcoma in an Indian peafowl (151) and lymphomas in captive greater and Attwater's prairie chickens (72, 78, 276).

The runting disease syndrome must be distinguished from Marek's disease in the chicken, especially when nerve lesions are also present. Differences between REV-induced and Marek's disease virus-induced nerve lesions have been discussed (263, 265), but are not consistent. Both types of nerve lesions must be distinguished from spontaneous neuropathy, a probable autoimmune lesion of peripheral nerves (24). Other immunodepressive diseases such as infectious bursal disease and infection with chicken anemia virus may also resemble the runting disease syndrome.

Chronic RE neoplasia in the turkey must be differentiated from lesions of lymphoproliferative disease of turkeys (108); differences in pathology and in properties of the viral reverse transcriptase can be helpful (205, 272). The PCR assays for lymphoproliferative disease (200) and RE (above) should also aid the differentiation of these diseases. Marek's disease has recently been diagnosed in turkeys in France, Israel, Germany and Ukraine (see subchapter on Marek's disease) and should be ruled out in the differential diagnosis of RE in turkeys.

In summary, naturally occurring RE lesions can be confused in the chicken with Marek's disease, lymphoid leukosis, and various other lymphoproliferative or immunodepressive conditions, and in the turkey, with lymphoproliferative disease virus-induced neoplasms and Marek's disease. An increasing awareness of REV-related syndromes, the increasing prevalence of REV infection, and the availability of improved diagnostic techniques should facilitate the inclusion of RE in the differential diagnosis of avian neoplasms.

Intervention Strategies

Vaccination

Although vaccines have not been seriously proposed for control of RE, some candidate vaccines have been described. Vaccination of chickens with a recombinant fowl pox virus expressing the *env* gene of REV (37, 168, 216), or empty REV particles produced by transfected QT35 quail cells (150), provided some protection against REV infection. Use of defective REV particles produced by infection of the D17 transformed canine cell line (250) has also been shown to induce neutralizing antibody in inoculated chickens (77). A baculovirus construct expressing the env gene of REV has also induced REV antibody in inoculated chickens (249).

Treatment

No treatment for RE is known. Since immune responses are mounted to infection, it is possible that some affected birds may recover.

Prevention and Control Procedures

Prevention of RE is currently accomplished through quality assurance of poultry biologics and, in SPF flocks, by strict biosecurity (257). Although it is desirable to prevent environmental exposure and seroconversion of breeder flocks where progeny are destined for export, this is difficult to accomplish because knowledge of the important natural reservoirs of infection is incomplete. Control of insect vectors and virulent fowl pox virus infections could be important in prevention programs (257).

Procedures for the control of RE have rarely been applied in commercial practice, mainly because the disease has been sporadic and self-limiting, but also because the necessary techniques and knowledge have not been available. Studies by Witter and Salter (266) on a flock of naturally infected breeder turkeys showed that REV has the potential to be a major economic problem, and provided an evaluation of some techniques for identification of shedder hens. Enzyme immunoassay to detect RE viral antigen in albumen samples seems to be the procedure of choice (109, 266). Presumably, it would be necessary to eliminate vertical transmission through removal of potential transmitter hens, and to rear progeny under isolated conditions whereby horizontal infection could be precluded. Many of these principles have successfully been applied to the control of avian leukosis virus in chickens. Such control procedures could be considered if REV infection becomes endemic in especially valuable breeding stock, as is the case with the endangered Attwater's prairie chickens (72, 78, 181, 257).

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Dermal Squamous Cell Carcinoma

Scott Hafner and Mark A. Goodwin

Introduction and History

There have been sporadic reports of squamous cell carcinomas in the skin, tongue, pharynx, crop, and esophagus of aged chickens worldwide since the 1800s. Squamous cell carcinomas in older chickens are rarely metastatic (1), but often are locally invasive (3, 7, 8, 14, 15, 18, 25, 27). In contrast, the term "dermal squamous cell carcinoma" (DSCC) has been used to denote lesions usually found in the skin of young broiler chicken carcasses at slaughter. This condition predominantly affects only the dermis, and there are no reports of metastasis (5, 6, 12, 13, 19, 20, 24, 26, 28).

In the past, DSCC has been accepted as being the appropriate name for these lesions in young broiler chickens (5,6,16,26); however, more recently authors have preferred to use either avian keratoacanthoma or keratoacanthoma (12,13,19,20). This is because keratoacanthomas are regressing tumors of humans that resemble squamous cell carcinomas both grossly and microscopically (2, 17), and naturally occurring DSCCs in live broiler chickens have also been found to regress (12).

Economic Significance

Carcasses with extensive lesions of dermal squamous cell carcinoma (avian keratoacanthoma) are condemned at slaughter, while less affected birds undergo trimming. Condemnations of whole carcasses represent a significant economic loss (11).

Public Health Significance

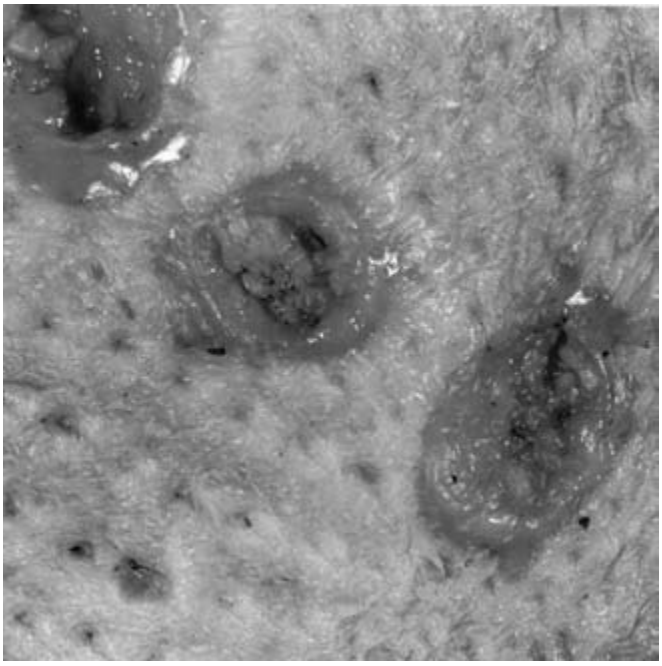
There is no known public health significance, although birds with extensive lesions may be septicemic due to secondary bacterial infections.

Incidence and Distribution

These skin lesions are most commonly found in broiler chicken carcasses, but similar tumors are also occasionally present in the carcasses of older chickens (12). In broilers, the prevalence of carcasses with multiple lesions averages from 0.01% to 0.05%, but may be 0.09% or higher in individual flocks (13, 16, 26, 28). Flocks of chickens slaughtered at less than 48 days had an increased prevalence of these skin lesions in one study (13) and in some investigations tumor prevalence was cyclic, lowest in summer months (13, 28). In some surveys, high condemnation rates were associated with dusty houses, birds placed in new houses, or with certain producers (11).

Etiology, Epidemiology, and Pathogenesis

The etiology of the natural condition is unknown. Applications of methylcholanthrene to chickens that were also chronically infected with fowlpox resulted in the formation of papillomas and squamous cell carcinomas; however, these tumors either regressed or resolved to cutaneous horns after applications of the carcinogen were discontinued. A few metastatic squamous cell carcinomas originated from residual lesions after several years had passed (9). In later work, repeated topical applications of methylcholanthrene produced regressing lesions that were originally diagnosed as squamous cell carcinomas (22), then as squamous cell carcinomatoid tumors (23), and finally as keratoacanthomas (21). More recently, DSCC developed in two young chickens that had been injected with strains of avian leukosis virus, but other similarly treated chickens did not develop these lesions (4). DNA sequences specific for fowlpox virus were detected by a nested PCR nearly as frequently in DSCC lesions as these sequences were detected in fowlpox lesions and much more frequently than these sequences were detected in normal skin or skin affected by dermatitis. However, no evidence of infectious poxvirus was identified (10).



15.56. Typical carcass lesions are craterous ulcers within feather tracts.

Gross Lesions

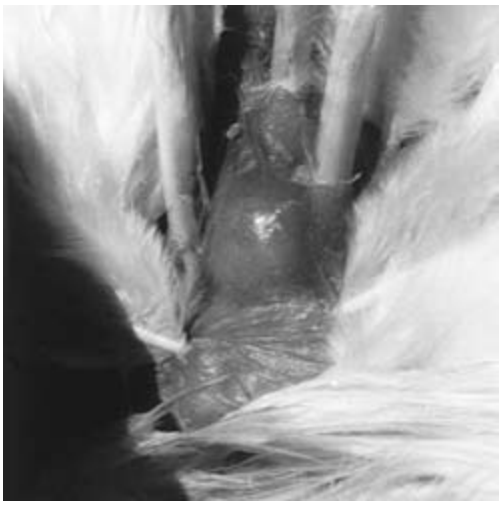
Carcasses most commonly exhibit crater-shaped ulcers with raised margins that occur in feather tracts (Fig. 15.56). Smaller ulcers average 5 mm in diameter and are circular, but large irregular, coalescing ulcers are present on some carcasses (13). These ulcerated lesions may either form aggregates within feather tracts, or they may be found scattered throughout the tracts. In one study (13), lesions occurred most frequently in dorsopelvic, femoral, and pectoral tracts, but some surveys have found no apparent site predilection (26). In live chickens, these ulcers are filled with keratin and cell debris (Fig. 15.57). Small (average 3 mm) nodular lesions often accompany ulcers and appear grossly as enlarged feather follicles (Fig. 15.58). In live young broiler chickens, these nodular lesions progressed to ulcers, and all lesions eventually regressed (12).

Histopathology

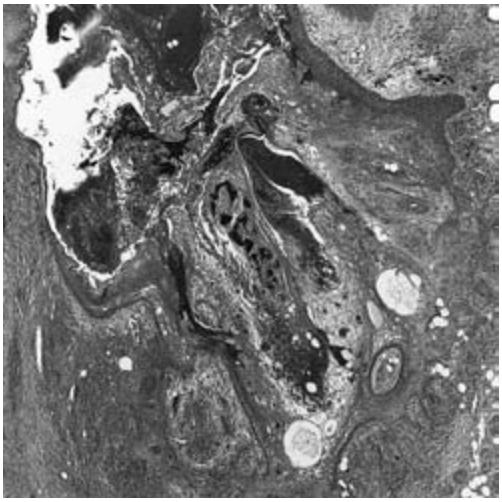
Nodular lesions appear microscopically either as proliferative outgrowths of feather follicle epithelium (Fig. 15.59), cysts that originate from dysplastic feather follicle epithelium, or hyperplastic feather follicles that contain hyperkeratotic feathers (13, 24). The cytokeratin profiles of tumor cells also suggest that tumors originate from the feather follicle epithelium rather than the surface epidermis (24). Ulcers are composed of a central cup-shaped cavity lined by epithelium and filled with keratin, bacteria, sloughed epithelial cells, and inflammatory cells. Epithelial lips overhang the central keratin mass. The lining epithelium keratinizes toward the central cavity and extends thin strands of keratinocytes into the surrounding dermal fibroplasia (Fig. 15.60). The peripheral fibroplasia contains isolated keratinocytes, het-



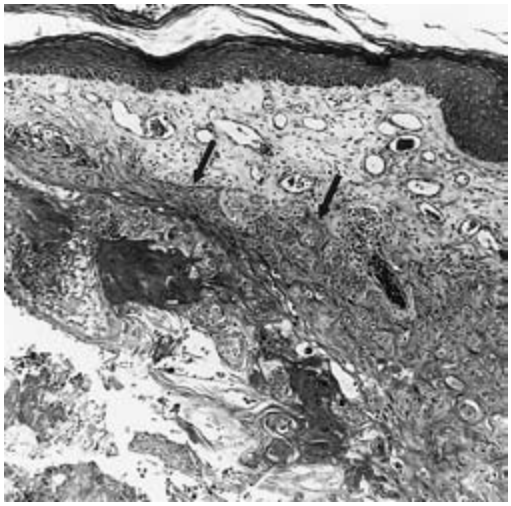
15.57. In live chickens, ulcers contain central masses that are mixtures of keratin, cell debris, and bacteria.



15.58. Early lesions in the skin of live chickens are nodules in the base of feather follicles.



15.59. Microscopically, nodular lesions are expansions of feather follicle epithelium. ×40



15.60. A section through the epithelial lip of an ulcer from a live bird shows the centrally keratinizing and peripherally invasive lining epithelium (arrows) adjacent to the central keratin and cell debris. ×100

erophils, scattered macrophages, and perivascular lymphocyte aggregates. Carcass lesions are often extensively altered by defeathering with loss of the central keratin core and much of the lining epithelium (12, 13).

Ultrastructurally, desmosomes interconnect keratinocytes that contain tonofibrils or keratohyalin granules. In some studies, viral particles were not seen (5, 12). However, in one study type C retroviruses were identified (24).

Pathogenesis

The complete pathogenesis of DSCC is unknown, however, lesions appear to begin at the margin of hyperplastic feather follicles. The peripheral follicular epithelium either infiltrates the adjacent dermis or expands into small cysts with irregular peripheral margins of invading keratinocytes. The lesions are further surrounded by a zone of fibroplasia that contains numerous inflammatory cells. As the cysts enlarge, they become superficially ulcerated. Overlying epithelial lips at the margins of the ulcers restrain the central mass of keratin and bacteria. After the central keratin core is lost, there is rapid regression of the lesion to a dermal scar overlaid by re-epithelialization.

Diagnosis

Grossly and microscopically, the differential diagnosis is primarily an ulcerative dermatitis. Adequate examination of microscopic sections confirms the diagnosis.

Prevention and Control

There are no known methods of prevention and control.

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Multicentric Histiocytosis

Scott Hafner and Mark A. Goodwin

Introduction

Multicentric histiocytosis is a condition of young broiler chickens that is characterized grossly by both splenomegaly and hepatomegaly with numerous small white masses in the spleen, liver, and kidneys. This condition has been noted since 1991 in the organs of young chickens presented for slaughter in the United States (4). Synonyms for this disease include "big spleen Marek's disease" and "reticuloendotheliosis-like syndrome." Similar diseases in other countries have been described as "histiocytic sarcomatosis" and "systemic spindle-cell proliferative disease" (1, 8, 9). It has not been determined whether these lesions are true neoplasms or a marked proliferative response (2, 4, 5, 6).

Public Health Significance

There is no known public health significance.

Etiology

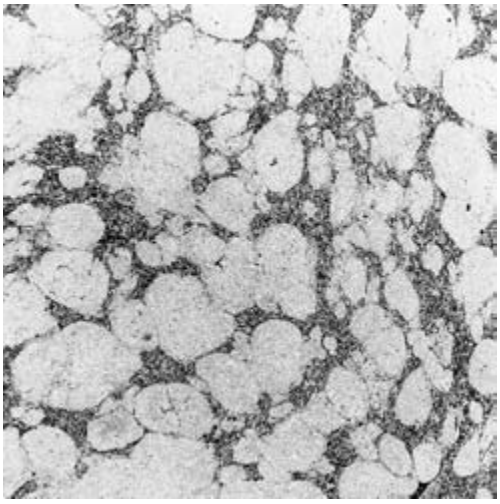
No definitive etiologic agent has been identified. In some examinations, the DNA extracted from lesions of naturally diseased broiler chickens did not contain sequences specific for reticuloendotheliosis viruses, Marek's disease virus, or exogenous leukosis-sarcoma viruses (4, 10), but subgroup J viruses may not have been detected by the primers used in PCR amplification of tumor cell DNA (1). Serum samples from naturally diseased flocks have not implicated Marek's herpesvirus, infectious bursal disease virus, or reoviruses (7). Broiler and SPF leghorn chickens injected with tissues from field cases have developed characteristic gross and microscopic lesions (3). In these birds, antibodies to REV or ALV did not develop and REV were not isolated, but six ALVs were isolated and grown in CEF resistant to ALV bearing the subgroup A envelope. However, only one of these isolates was identified by PCR of the CEF culture DNA as a subgroup J ALV. In a British study rare lesions termed "histiocytic sarcomatosis" were detected in meat-type chickens experimentally infected with HPRS-103 or related strains of subgroup J ALV at 1–2 days of age, but not in similarly infected leghorn chickens. Lesions were determined to be composed of a mixture of macrophages, dendritic cells, and lymphocytes (1). In Japan, broiler chicken carcasses condemned for suspicion of Marek's disease were determined after microscopic examination to contain multiple organs affected by proliferative lesions composed of spindle cells. Avian leukosis virus antigens were detected in these spindle cells and evidence of subgroup J ALV infection was detected by PCR (8, 9).

Gross Lesions

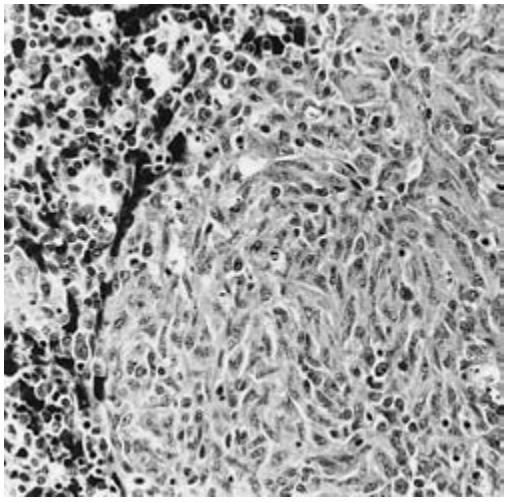
Spleens are enlarged 2–4 times normal and livers are enlarged (2×) normal size. Miliary (0.5–2 mm) white-to-yellow nodules are present throughout the spleen and liver. Similar 1–5 mm nodules are often visible in the kidney, but gross changes are rarely identified in the other organs. Some diseased birds are pale and are smaller than their flock mates (2, 4, 5, 6). Lesions are not accompanied by myeloid leukosis (myelocytomatosis) (3).

Histopathology

In the spleen, circular nodules of spindle-shaped cells diffusely expand periarteriolar lymphoid sheaths (Fig. 15.61). These histiocytic cells (denoting cells with abundant eosinophilic cytoplasm) contain elongated oval, fusiform, or more bizarrely configured nuclei (Fig. 15.62). Mitotic figures and individually necrotic cells are common in these nodules. The exact lineage of these cells has not been identified by specific markers although cells resemble fixed macrophages or dendritic cells. Multinucleated cells are not present. A few plasma cells, small lymphocytes, scattered lymphoblasts, and germinal centers are often present within nodules. Similar but often more heterogenous nodules diffusely stipple the liver, primarily replacing periportal hepatocytes and bulging into portal veins. Nodules also partially replace the bone marrow, kidneys, pancreas, proventricular glands, and lungs. There are diffuse accumulations of histiocytic cells under the mucosal epithelium of the proventriculus and in the enteric mucosal lamina propria. In the duodenum these cells fill the lamina propria and may expand deep into the muscularis. Occasionally perivascular aggregates of lymphoblasts are present in the musculature of the ventriculus and in cardiac and skeletal muscle (4).



15.61. Nodules diffusely expand the splenic periarteriolar lymphoid sheaths. ×20



15.62. Histiocytic spindle-shaped cells contain elongated and pleomorphic nuclei. ×200

Diagnosis

Histopathology is currently the only method of diagnosis. Nodules are characteristic and are consistently found within specific locations in multiple organs (4).

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Other Tumors of Unknown Etiology

Rodney L. Reece

Introduction

Standard veterinary texts dealing with general pathology or neoplasia rarely mention tumors of poultry, and the most comprehensive description of tumors of the domestic fowl remains that of Campbell (25), even though it is out of print. The purpose of this chapter is to provide an outline of the more commonly encountered tumors of unknown etiology of poultry. In this respect, much is owed to accounts by other veterinary pathologists (48, 63, 66, 81, 93, 106). Where relevant, reference is made to tumors in other avian species because their pathogenesis is expected to be similar to that of equivalent tumors in poultry, and there is a good morphological correspondence between many avian and mammalian tumors thus allowing some extrapolation of observations on mammalian tumors. As with mammalian species, the histologic appearance of avian tumors allows most to be classified according to their cell of origin. Detailed studies involving tinctorial charateristics with special stains, cytogenetics, immunohistochemistry, and electron microscopy are useful for more accurate classification, but such techniques are rarely applied to poultry tumors because of the lack of incentive and resources to investigate what are deemed to be incidental conditions discovered during investigations of flock problems.

Prognosis and treatment are not discussed in this chapter. In human and companion animal pathology immunoreactivity of tumors to various markers of cell type and function are undertaken as prognostic indicators and guides to appropriate therapeutic interventions. In avian medicine and particularly with regards to commercial poultry such studies are rare, but they are becoming more prevalent in companion bird medicine where there is a high individual monetary and emotional value, and a relatively long life for animals in good health. Specific staining reactivity with many monoclonal antibodies decreases significantly if tissue is held in fixative for more than 48 hours. Samples several days in transit to the laboratory and archival materials can yield uninterpretable, rather than negative, results. Additionally caution needs to be exercised in the interpretation of immunohistochemistry in avian species using antibody raised against mammalian proteins, and different antibody preparations intrinsically may have very different reactivities. Some tumors may lose specific immunostaining capacity due to anaplasia. In any test schedule appropriate positive and negative controls are necessary.

Some studies have successfully utilized antibodies to identify tissues in avian species, both neoplastic and normal: vimentin is found in most mesenchymal cells (11), desmin occurs in smooth and striated muscle fibers (11), cytokeratin, of which there are many types, in epithelial cells (99), actin in muscle fibers (110), neurone-specific enolase in neuroepithelial cells (79), anti-neurofilament-200 in nerve fibers (140), anti-myelin basic protein in Schwann cells (19), and in situ detection of hormones such as growth hormone (140). The S-100 protein family contains a number of related calcium-binding proteins and they are found in variety of tissues. Mammalian cutaneous melanocytes contain a large amount of S-100-A and S-100-B, and thus immunoreactivity is used as a marker of melanoma. In lower vertebrates the melanocytes appear not to contain epitopes recognized by some antibodies raised against mammalian S-100 (89). Additionally the range of tissues staining in avian species with antibody derived from mammalian S-100 appears to vary: antibody to S-100 protein did not stain nerve elements in white Pekin ducks (142) but did so in other avian species (89, 140).

The proliferative activity of a tumor may be defined by the number of mitotic figures per high power field in densely cellu-

lar types of tumors. More quantitative assessments can be made by other methods including immunohistochemistry using cell cycle specific monoclonal antibodies such as against proliferating cell nuclear antigen (PCNA). The amount of PCNA rises during the S-phase of cell division but the half-life of PCNA is shorter in cells that cycle compared to those that move into a quiescent state and interpretation needs to take that into account. A few studies of avian neoplasms have utilized PCNA (for example 99).

One of the best characterized tumor suppressor genes is p53. This gene encodes a nuclear phosphoprotein that regulates the movement of the cell through the cell cycle. Mutation of the p53 tumor suppressor gene is a common genetic alteration in human and other mammalian tumors. The monocloncal antibody PAb-240, which is specific for both mutant and wild types of mouse p53, cross-reacts with chicken p53 and has been shown to have over expression in virus-induced lymphomas in chickens (59). The role of p53 in other avian tumors has yet to be determined.

In the study of avian tumors, attention has been focused on those of viral etiology, both from the standpoint of their economic importance and as potential models applicable to tumors in humans (21). Tumors of the reproductive tract of laying hens, keratoacanthomas of broiler chickens, and amputation neuromas have been studied to some extent, but little research has been directed to the other neoplastic diseases of unknown etiology in poultry. The recognition of avian leukosis virus serotype J infection (see "Leucosis/Sarcoma") as a cause of what were traditionally considered non-virally induced tumors such as granulosa cell tumors, mesothelioma and pancreatic adenocarcinoma (111) may yet help to provide a greater understanding of tumor pathogenesis.

The incidence of non-virally induced tumors appears to be low, but properly constructed surveys are needed to clarify the situation. In a report of cage layers inspected at Irish abattoirs, the condemnation rate was 1.4%, of which one-fifth (0.3%) was due to nodules; 90% of the nodules were tumors and 70% (<0.2%) of these were adenocarcinomas, probably derived from the reproductive tract (145). A histological study of skin lesions in downgraded broiler carcasses concluded that accurate data required histological examination because changes observed grossly were not necessarily specific (46). Caution needs to be exercised in interpreting results of such surveys because early or small tumors are unlikely to be detected, organs such as the brain and the oviduct lumen are not routinely examined, and histological examination of the skin is rendered difficult because of processes at the abattoirs.

Official animal health surveillance in many countries has become more focused on early identification of notifiable diseases, detection of new and emerging diseases of possible animal and human health significance, identification of diseases or problems likely to compromise animal welfare, and monitoring of changes in selected endemic diseases which could effect efficiency and/or productivity of livestock. Combined with a move towards cost recovery, there has therefore arisen a significant disincentive to submit routine diagnostic material to government veterinary pathology laboratories. In the six months following the outbreak of high pathogenicity avian influenza in New South Wales, Australia 1997, many poultry were necropsied to determine the cause of death or respiratory distress. Three cases of oropharyngeal squamous cell carcinoma and one case of gizzard adenocarcinoma were observed (122). These birds were all noncommercial adults more than a year old from unrelated flocks and of different breeds. In the surveillance following the outbreak of virulent neurotrophic Newcastle disease virus in New South Wales, Australia, 1998–2000, the brains and tracheas of many poultry exhibiting nervous signs or found dead were examined histologically. These birds were predominately broilers and therefore of a relatively young age. A single slightly off midsagittal section of the brain and brain stem, and a single transverse section of the upper trachea, were histologically examined. Some of the samples submitted were of poor quality but were adequate for exclusion of Newcastle disease. Brain tumors were not observed. One enterprising veterinarian included a subcutaneous nodule from an unspecified site of a 5-week-old broiler that was diagnosed as a schwannona, and an unrelated 6-week-old broiler had a tracheal leiomyoma (122). In addition, two cases of cystadenoma involving the infra-orbital sinus were found in adult noncommercial poultry, one hen and one cockerel. These tumor types had not been observed previously at this laboratory, nor by the author elsewhere, nor since. As these types of tumors were present in the population but not previously detected, it would appear that the incidence of tumors of unknown etiology in the wider poultry population cannot be ascertained with any confidence by examination of records from passive surveillance.

The life span of commercially raised chickens and turkeys is generally short and may be less than that required for development of some non-virally induced tumors. The restricted information available on tumor incidence in older birds (the potential life span of chickens is generally considered to be around 15 yr but may be up to 35 yr) comes from several sources. The first is represented by long-term studies of flocks of aged chickens (51). The second is from diagnostic reports by veterinary pathologists, particularly of poultry maintained for periods of time longer than those of poultry kept under intensive conditions (134, 135). The third comes from necropsy reports from zoos where various species of birds often are maintained for natural life spans and are usually necropsied at death (30, 43, 78, 96, 98, 102, 118), and also from studies on other captive and wild birds (17, 34, 119, 125, 132). Reports of neoplastic diseases in the latter category have provided useful information, albeit not directly applicable to poultry. In particular, captive budgerigars (*Melopsittacus undulatus*) have a high incidence of tumors, although there is some evidence of retrovirus infection in this species, which could be partly responsible (64). The incidence of tumors in wild budgerigars is not known.

This chapter includes personal observations of spontaneous neoplasms in a U.S.A. flock of 466 specific pathogen free (S.P.F.) white leghorn hens, many of which were allowed to live out their natural life span (51); moribund and dead birds from an Australian S.P.F. flock; and field cases submitted for necropsy. The U.S.A. S.P.F. flock was free of clinical Marek's disease and exogenous avian leukosis virus, and the following tumors were diagnosed: 142 ovarian tumors (adenocarcinomas, granulosa cell tumors, and ovarian Sertoli cell tumors), 40 oviductal tumors

(adenocarcinomas and leiomyomas), seven pancreatic adenocarcinomas, and one case each of parabronchial adenocarcinoma, proventricular adenocarcinoma, hepatocellular carcinoma, cholangiocellular carcinoma, and mesothelioma (52). It is not certain, however, how applicable these rates of spontaneous neoplasms are to other breeds and strains of chickens, since this strain had a high incidence of genital tumors for which there is some genetic predisposition (see "Reproductive System"). The Australian S.P.F. flock was free of Marek's disease virus, exogenous avian leukosis virus, and reticuloendotheliosis virus and the following tumors were identified: two cases each of lymphomas, fibrosarcomas, and metastatic abdominal adenocarcinomas, and one case each of myelocytoma, reticulum cell sarcoma, histiocytic sarcoma, abdominal liposarcoma, subcutaneous lipoma, renal adenocarcinoma, granulosa cell tumor, and adrenocortical adenoma (121). In neither flock were these tumors associated with known oncogenic viruses.

There are reports of lymphoid tumors in S.P.F. chickens (37, 121), which indicates that on occasions, such tumors may be induced by factors besides known transforming viruses. Additionally, lymphomas are a common diagnosis in other avian species (97, 119, 150) and on occasions they have been inappropriately described as Marek's disease or lymphoid leukosis without direct evidence of involvement of transforming viruses. It would be preferable for such cases to be described in morphologic terms (for example lymphosarcoma or mixed cell lymphoma) rather than ascribing them a name denoting etiology. The older surveys and reports of poultry tumors cited in this review (22, 63, 81, 106) were published prior to the implementation of control programs for Marek's disease and lymphoid leukosis. After allowing for cases likely to have a viral etiology, the prevalence of other tumors was low, with the overwhelming majority being derived from the reproductive tract of adult hens. That situation still appears to prevail.

Public Health Significance

The tumors described in this section mostly occur at extremely low prevalence. The only exception is tumors of the female reproductive tract of adult hens and there is evidence that this may be related to selection for mature body weight and egg production (3): it is not related to use of female hormones. Contrary to the common urban myth commercial poultry are not treated with growth inducing or gender associated hormones and therefore there is no potential public health risk related to that. Tumors of the female reproductive tract are not present in commercially killed broiler chickens because of their young age. Advanced cases in spent layers and breeders are unlikely to enter the human food chain as the affected birds are not suitable for killing in abattoirs and/or the carcasses will be condemned. Hens with oviductal adenocarcinomas cease laying eggs.

Reproductive System

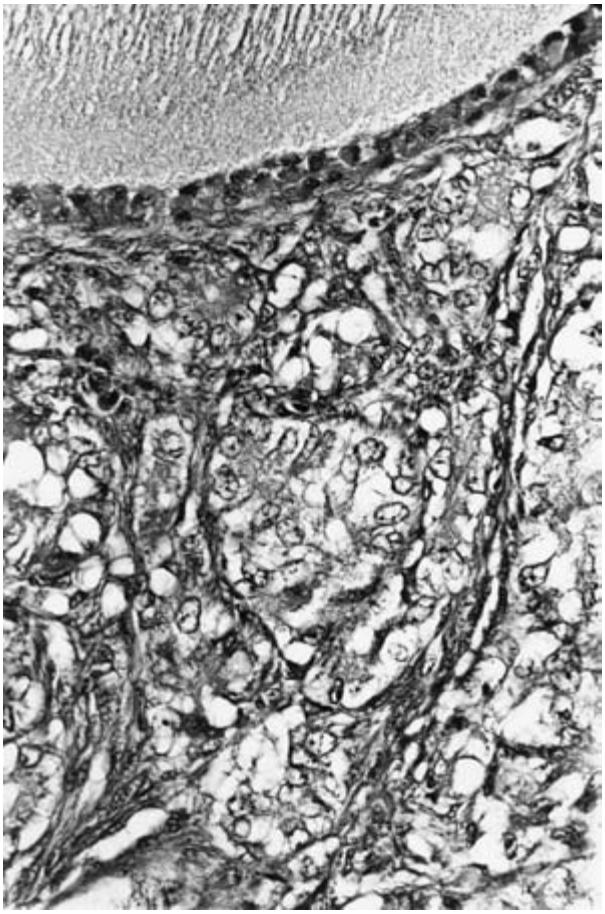
Ovary

The classification of gonadal tumors of poultry is complex and controversial. It is somewhat complicated because of a tendency for investigators to apply to avian species terms used in the description of mammalian, and particularly human, ovarian tumors. This ignores the dissimilarity between mammalian and avian ovaries in terms of histology, endocrinology, and physiology. Some authors have argued that avian gonadal tumors should be defined no further than gonadal tumors (for example 17) until a great deal more is known about them. This would solve some problems but create others. It is sometimes useful to consider ovarian tumors as being derived from surface mesothelium (adenocarcinomas); sex cord (granulosa-theca cell tumors and arrhenomas); germ cell (dysgerminomas and teratomas); or arising from connective tissue elements, other supportive tissues or as metastases from other sites or tissues (fibrosarcomas and lymphosarcomas). It should be noted that myxomas and fibromas of the ovary may, on gross examination, be mistaken for adenocarcinomas, and both fibrosarcomas and myxosarcomas can occur as metastatic abdominal tumors requiring histologic study to differentiate them from metastatic abdominal adenocarcinomas. Ovarian tumors have been described in turkeys (151), budgerigars (8) and other avian species (119). In hens, ovarian tumors are usually observed in birds more than 1 year old.

Adenocarcinoma

Early tumors are small, round, white, and firm nodules on the ovarian surface, which may be mistaken for atretic follicles. In advanced cases, these coalesce into a gray-white, firm cauliflower-like mass. Numerous transcoelomic implants are common at this stage, varying from small pearl-like growths to massive nodular tumors on serosal surfaces of the pancreas, oviduct, mesentery, and intestines. Ascites usually develops when such tumorous growth is extensive. The walls of affected intestines are thickened and adhered together, and the intestinal lumina become constricted. Metastatic abdominal adenocarcinomas may originate from either the ovary or the oviduct, and differentiation can be difficult, as in either case the ovary and the oviduct may be involved. Many cases of metastatic abdominal adenocarcinomas are described as ovarian adenocarcinomas without any serious attempt to determine their origin. Failure to detect tumor growth in the mucosal lining of the oviduct indicates that the tumor was not of oviductal origin and, therefore, probably arose from the ovary. For confirmation, frozen tissues can be stained immunohistologically for ovalbumin, which is only present in tumors arising from the magnum of the oviduct (72). Terminally hens are extremely thin and assume an upright, penguin-like position. Usually there are no maturing follicles in advanced cases, and the oviducts are inactive.

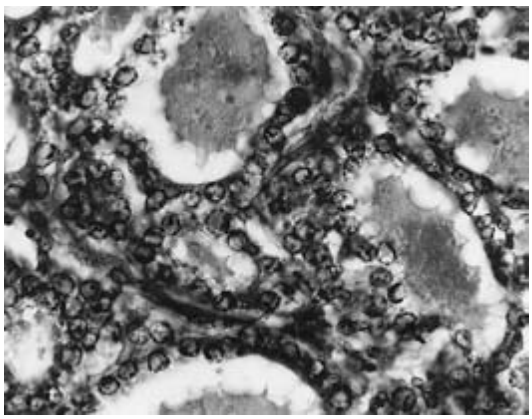
The cell of origin of these tumors remains to be identified but is frequently assumed to be the overlying mesothelium (so-called germinal epithelium) of the ovary or its invaginations into the ovarian cortex; alternatively they may be derived from thecal glands, interstitial cells, remnants of embryonic sex cords, or the mesonephros. The tumor may start in the theca externa of smaller follicles (Fig. 15.63), in the interfollicular stroma or occasionally fairly deep in the ovarian stalk. They often are multifocal in origin, but growth is fairly slow over a period of months. Ovarian adenocarcinomas are not associated with excess production of steroidal hormones (51).



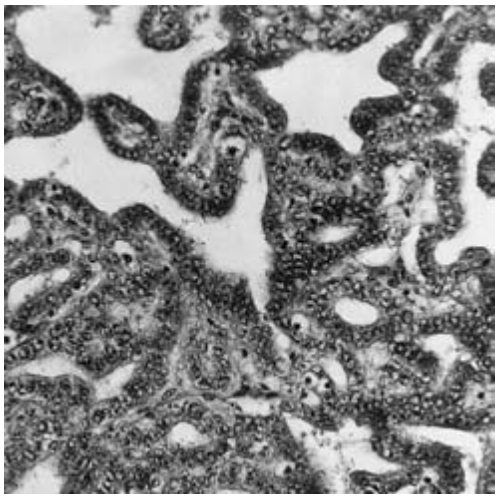
15.63. Ovarian adenocarcinoma in the theca region demonstrating delicate trabeculae and round nuclei; note the granulosa cells and yolk of the developing ova (top). H & E, ×360.

Histologically, the commonest structures comprising the ovarian adenocarcinoma are acini formed by a single layer of low columnar or cuboidal non-ciliated epithelium. These eosinophilic cells with basal, round nuclei are oriented around a lumen of variable size and shape, sometimes containing an intensely eosinophilic, homogeneous material that is periodic acid-Schiff (PAS)-positive and mucicarmine-negative (Fig. 15.64). Other tumors are more densely cellular, with the acinar structures compressed to give the appearance of islands or sheets of tumor cells, while in another variant, the lumen may be enlarged with infolding of the neoplastic lining forming papillary structures (Fig. 15.65).

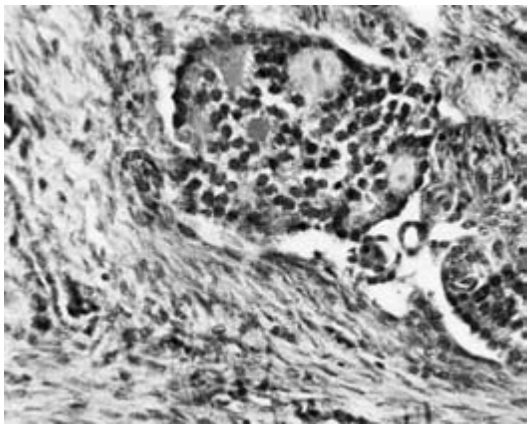
The prevalence of mitotic figures varies from scant to abundant, although in most cases they are not prominent. Division of ovarian adenocarcinomas into medullary or scirrhous forms appears unwarranted, as size determines morphology; the acini of large tumors are interlaced with dense fibrous tissue (Fig. 15.66), whereas smaller tumors have a lesser component of fibrous tissue. Serosal implants may induce a proliferative response of smooth muscle in the underlying muscularis, but the extent of this varies (104). Ovarian adenocarcinomas similar to those seen in the chicken have been described in mature turkey hens (151).



15.64. Acinar structures, typical of ovarian adenocarcinoma filled with eosinophilic material and lined by cuboidal cells containing round nuclei with condensed chromatin and sparse eosinophilic cytoplasm. H & E, ×600.



15.65. Ovarian adenocarcinoma with papillary structures projecting into dilated acini. H & E, ×160.



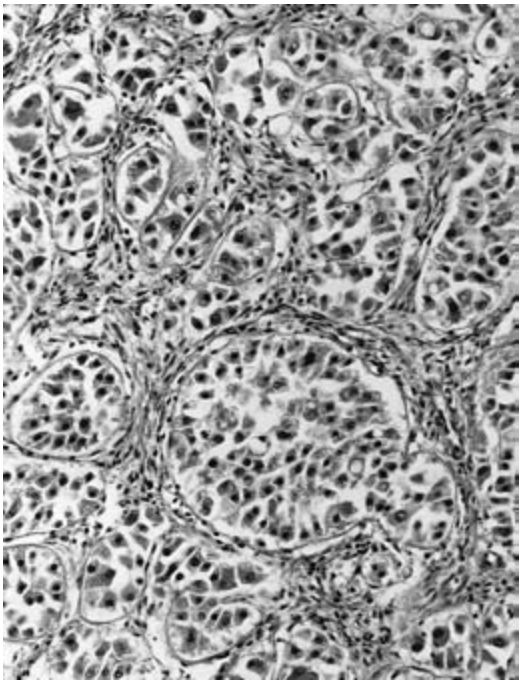
15.66. Another form of ovarian adenocarcinoma with dense bands of stromal cells enclosing clusters of neoplastic acinar cells with intensely basophilic nuclei. H & E, ×160.

Occasionally ovarian adenocarcinomas are found in ovaries covered with grape-like clusters of follicles filled with yellow fluid. Some of these are cystadenocarcinomas comparable to ovarian cystadenocarcinomas of mammals. In others the cystic spaces are lined by flattened stromal lacunae cells. In this context it is relevant to remember that the avian ovary has a well-developed anastomosing stromal lacunae system, the lining cells of which have phagocytic properties and resemble peritoneal lining mesothelial cells. These lacunae are not directly connected to the vascular or lymphatic systems but are actively involved in removal of burst ovarian follicle yolk and other fluid and thus may become involved in adenocarcinomas. Cystic ovarian follicles occur in other avian species and are not related to neoplasia (75). Large cystic acini lined by low cuboidal to squamous epithelium may be found in some cases of metastatic abdominal adenocarcinomas, and their lumina contain a PAS-positive mucinous secretion similar to that described above (25, 121). Ovarian myxomas occur and they may present with tenacious mucinous material exuding from the cut surface, but histologically they are quite distinctive.

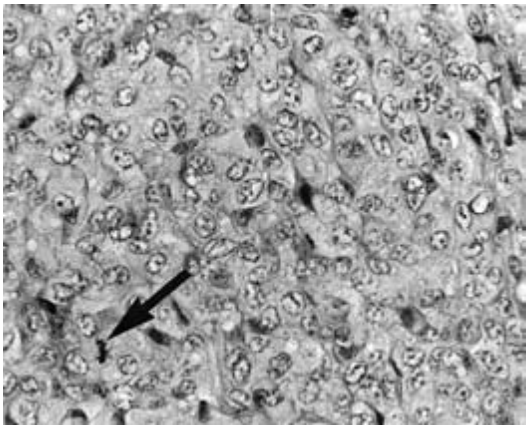
Granulosa-Theca Cell Tumor

This tumor is yellow, round, and lobulated with an extremely friable consistency very different from the firm, cauliflower-like adenocarcinoma. Granulosa-theca cell tumors are encapsulated within a smooth, glistening membrane, and larger tumors have extensive areas of necrosis and hemorrhage. Tumors that are attached to the ovary only by a thin stalk may grow to enormous size, and metastasis to adjacent viscera occurs occasionally. Histologically these tumors are composed of pale, eosinophilic, polyhedral to fusiform cells with some cytoplasmic vacuolation (Fig. 15.67). The arrangement of these cells can be very variable even within a single tumor. They may form tubular, or less frequently, follicular structures (Fig. 15.68), separated by a delicate vascular stroma. In some cases there may be elaborate cylindriform or gyriform arrangements (Fig. 15.69), or typical rosettes of groups of a dozen or so epithelial cells clustered radially around small central spaces (Fig. 15.70). In others the stroma may be prominent. The proportion of mitotic figures varies but tends to be low, and the tumor appears to grow at a slow rate.

The tumor cells have been confirmed as granulosa cells because they have an ultrastructural component known as the transosome, which has been identified solely in avian follicular granulosa cells (77). Greatly elevated plasma concentrations of estrogen are found in hens with large granulosa-theca cell tumors (51). It is known that granulosa cells from mature follicles normally produce progesterone, whereas theca cells produce estrogen (112). This, coupled with the observation of numerous theca glands in granulosa cell tumors, justifies the binomial descriptor granulosa-theca cell tumor. The high concentrations of circulating estrogen result in the oviducts being similar in size to those of laying hens. Comb development is as for hens in lay, but eggs are not produced. The existence of separate ovarian thecal cell tumors such as those described by Campbell (25) still awaits further study. The presence of highly vacuolated theca-like cells may lead to the tumor being designated as luteinized and if this is excessive, the tumor may be referred to as a luteoma.



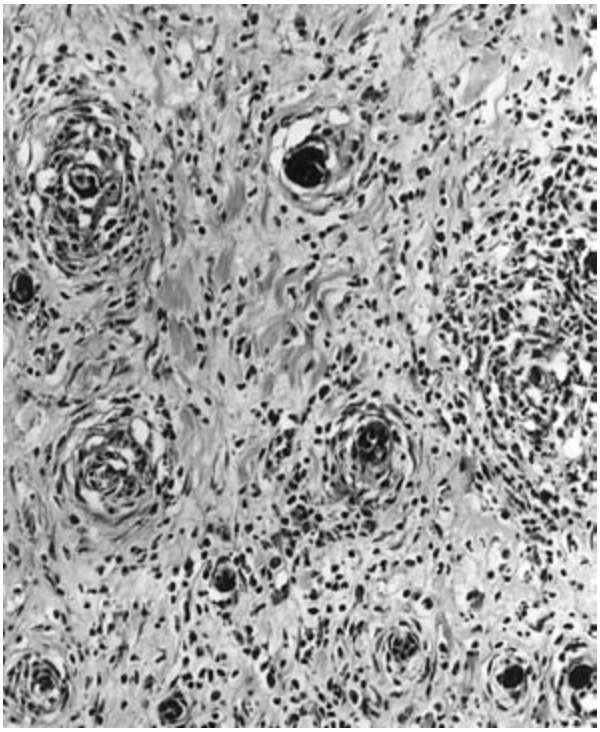
15.67. Lobules of vacuolated epithelial cells separated by moderate trabeculae in a granulosa-theca cell tumor. The central lumina are not as definite as in adenocarcinomas. H & E, ×140.



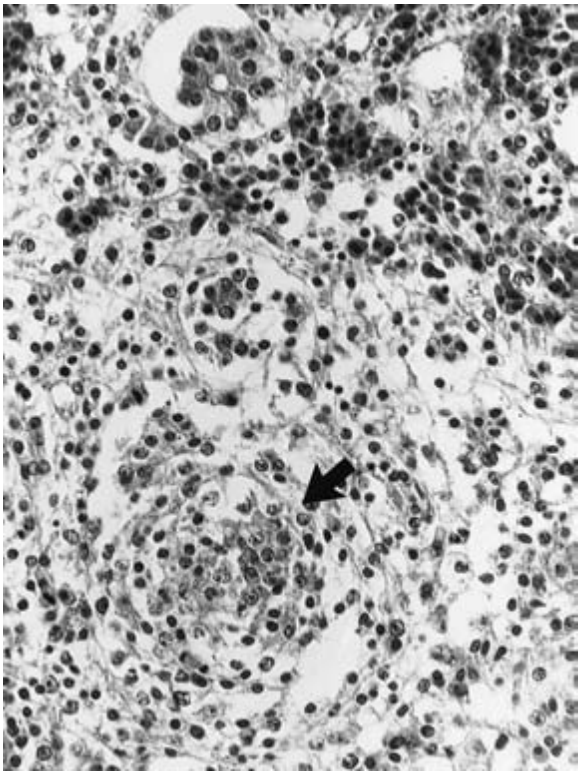
15.68. Granulosa-theca cell tumor composed of a uniform population of tightly packed tumor cells with plentiful, pale eosinophilic cytoplasm and uniform, round vesicular nuclei. Note the mitotic figure (arrow). H & E, ×600.

Arrhenoma and Arrhenoblastoma

The terms arrhenoma and arrhenoblastoma may be applied in a morphologic sense to ovarian tumors with testicular elements, or in a functional sense, to encompass a diverse group of virilizing ovarian tumors. Sex reversal (virilism) in domestic fowls has been recognized since ancient times (50), but very few of such cases are due to ovarian tumors (25). It must be remembered that in avian species, contrary to the situation in mammals, the male



15.69. Gyriform arrangements of cells in one area of an ovary with a granulosa-theca cell tumor. H & E, ×90. (Courtesy of *Avian Pathology*)



15.70. Granulosa-theca cell tumor showing tubular arrangements and rosettes formed by clusters of cells radiating out from small central lumina. H & E, ×365. (Courtesy of *Avian Pathology*)

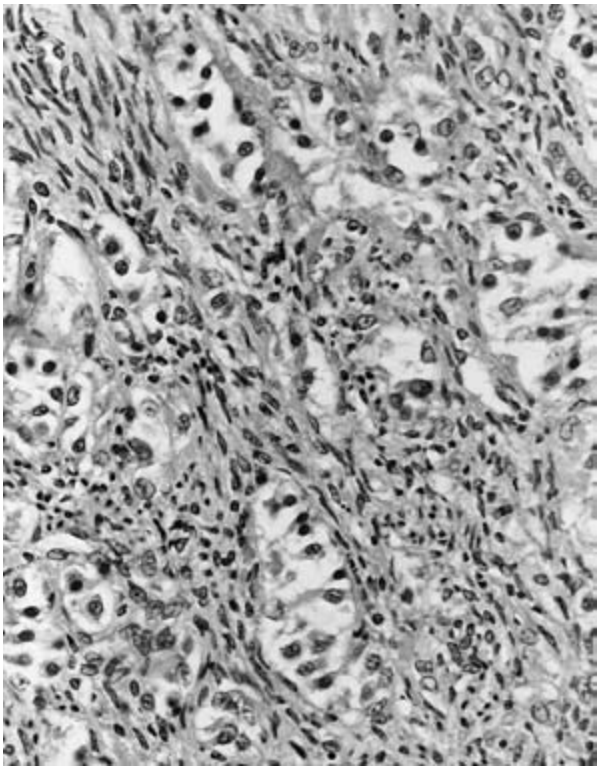
is the neutral sex and the female chick is demasculinized by her ovarian hormones (107). In the hen only the left ovary normally develops, but rudimentary male medullary tissue and primordial cells are present in the normal ovary (57). Surgical removal of the functional ovary leads to hypertrophy of the vestigial right gonad into an organ resembling an ovo-testis or testis, depending upon the age at treatment. The ovo-testis so formed has some areas of immature seminiferous tubules, but spermatogenesis is not normally a feature (20). Destruction of the left ovary by a non-steroid-producing tumor or other pathologic processes may result in the formation of a right ovo-testis. In studies of sex reversal in the fowl there is one well-documented case of an adult hen that laid eggs but subsequently developed ovarian pathology, underwent sex reversal, and was able to successfully fertilize eggs. However this was a case of tubercular oophoritis, not neoplasia (49). The opposite situation of feminization is very poorly documented (see "Sertoli Cell Tumor").

In this chapter the terms arrhenoma and arrhenoblastoma are reserved for those cases in which there is an ovarian tumor associated with some evidence of sex reversal (virilism). There are no reports on hormone production of these tumors in poultry, so it is not known if sex reversal is due to a lack of estrogens or the production of androgens. Arrhenomas are characterized by growth of seminiferous tubules within the ovarian stroma and appear as white, solid, lobulated masses within atrophic ovaries. They are of uncertain histogenesis and histologically are extremely variable. In the most differentiated form they are composed of branching cords of columnar epithelium, often two cell layers deep, which resemble immature seminiferous tubules. Spermatogenesis tends to be absent or poor. A loose or compact network of fusiform and epithelial cells arranged as cords, nests, rosettes, or incomplete tubules may be observed in less well-developed forms (Fig. 15.71). The interstitium may be prominent and contain nests of polyhedral lipoid-rich cells resembling Leydig cells. The seminiferous-like tubules can be filled with vacuolated cells (68). In large tumors there may be cystic cavitation and hemorrhage. Experimental induction of masculinizing arrhenoblastomas by injection of radioactive isotopes into the left ovary has been described (152). Arrhenomas may be mistaken for adenocarcinomas.

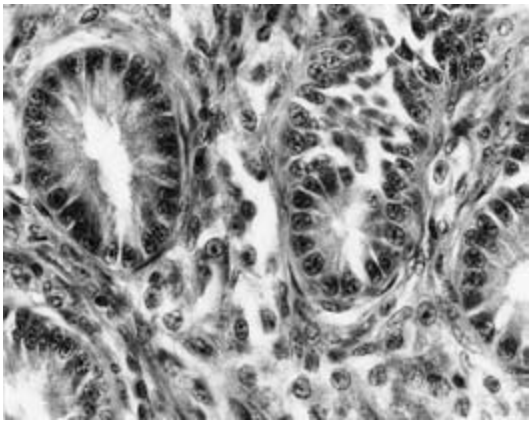
Gynandroblastomas are mixed steroid-producing tumors with estrogens produced by granulosa-theca cell components and androgens produced by arrhenomatous tissue.

Ovarian Sertoli Cell Tumors

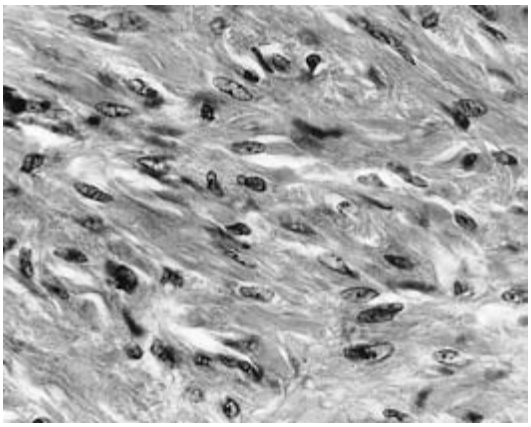
In the five cases of ovarian Sertoli cell tumors reported by Fredrickson (51), obvious sex reversal was not apparent, and circulating hormone concentrations were comparable to those of non-laying hens. Compact masses of tubules developing multifocally under the ovarian capsule were seen histologically. Interstitial cells were variably present, and the well-defined tubules lined by a single layer of columnar epithelial cells with basal nu-



15.71. Arrhenoma from a hen that showed sex reversal. Network of epithelial cells arranged as ill-defined cords and tubules. H & E, ×350. (C. J. Randall)



15.72. Ovarian Sertoli cell tumor composed of well-defined seminiferous-like tubules lined by Sertoli cells. Stroma contains interstitial cells. H & E, ×600.



15.73. Leiomyoma of mesosalpinx composed of smooth muscle fibers arranged in compact whorls. Mitoses are absent from this field, and the nuclear/cytoplasmic ratio is low. H & E, ×600.

clei were considered to be Sertoli cells (Fig. 15.72). Some ovarian Sertoli cell tumors appeared to develop within granulosa-theca cell tumors.

Dysgerminoma

Wight (155, 156) detected four ovarian tumors in pseudohermaphrodites, and three of these were designated as dysgerminomas, the equivalent of ovarian seminomas. These were not associated with sex reversal, but rather with loss of external morphologic features of hens and acquisition of some male characteristics such as enlarged combs and male-type saddle feathers. These tumors are considered to originate from seminiferous elements within the left ovary or vestigial right gonad. Histologically they consist of elaborate fibrous trabeculae surrounding cords or groups of round or polygonal cells, and occasional syncytia.

Mesosalpinx

Leiomyoma

Leiomyoma of the mesosalpinx is a common tumor in hens. They are usually located centrally in the ventral ligament of the oviduct, an area normally rich in smooth muscle. Occasionally, leiomyomas may be found on the peritoneal surface of the oviduct or growing in the mesentery. They vary from small white nodules to large gray heavily vascularized masses several centimeters in diameter. This tumor is usually a single, sharply circumscribed, encapsulated, solid, round mass with a characteristic white, glistening appearance on the cut surface. They are benign and composed of interlacing bundles of smooth muscle separated into fasciculi by a variable component of fibrous tissue (Fig. 15.73). These tumors may be referred to as leiomyofibromas or fibroleiomyomas, depending upon which tissue predominates. Mitotic figures are rare. They appear to have little effect on the oviduct or its function, although they may predispose to ova escaping into the abdominal cavity. The prevalence of this tumor in different strains of S.P.F. and commercial hens varied from 0–60% at the end of their first year of lay (5). Affected hens had elevated concentrations of circulating 17-ß-estradiol (4) and a high incidence of these tumors was induced in a commercial white leghorn strain by treatment with both diethyl-stilbestrol and progesterone, thus confirming a role for these steroid hormones in tumorigenesis (5).

Oviduct

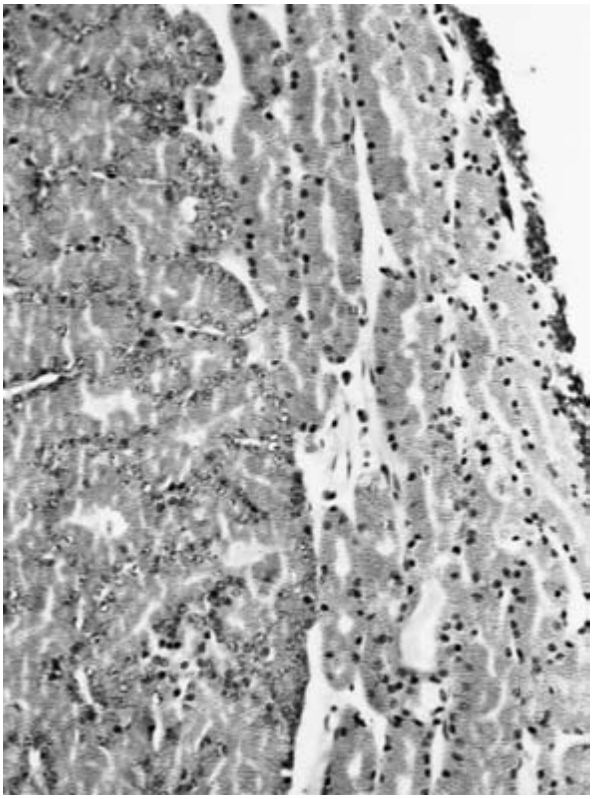
Adenocarcinoma

Most adenocarcinomas of the oviduct originate in the upper magnal portion of the oviduct, with occasional cases occurring in the infundibulum or uterus (shell-gland). Large focal and abdominal metastatic tumors are usually detected in hens more than 1 year of age. In a survey published in 1969 (60), the prevalence of oviductal adenocarcinomas in end-of-lay hens, determined by examining the mucosa of oviducts, varied from 5 to 81%. A positive correlation was found between tumor incidence, and mature body weight and egg weight (3). This indicates the possibility of an association with selection for egg laying, but properly constructed surveys need to be carried out. Metastatic abdominal adenocarcinomas observed at necropsy or abattoir inspection are but a small proportion of actual cases of oviductal adenomas and adenocarcinomas, and some such adenocarcinomas may be of ovarian origin. If the organ of origin is not readily determined, it would be preferable to refer to them as metastatic abdominal adenocarcinomas of unknown origin.

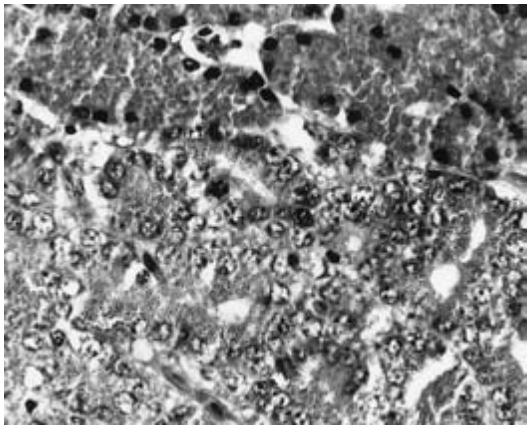
Studies of adenomas and adenocarcinomas of the magnum of the oviduct in domestic fowls and turkeys revealed a progression from focal dysplasia through sessile clusters to polypoid masses. The earliest lesions are small (2–10 mm in diameter) nodules on the ridges of the glands and may be found in laying hens at 30 wk of age. They are easily overlooked (143). Histologically these early nodules are composed of closely packed columnar cells with secretory granules in the apical cytoplasm and pale nuclei. The cells are oriented concentrically rather than toward the lumen (Fig. 15.74). These lesions are probably pre-neoplastic. Their incidence in commercial poultry is not known.

Individual or clustered sessile adenocarcinomas are gray and firm. They tend to coalesce into large, irregularly shaped tumors protruding into the oviductal lumen. Early lesions are found in hens with active ovaries, whereas abdominal metastases are associated with ascites and loss of bodily condition. In the primary tumor in the magnum there is generally a distinct boundary between neoplastic and normal glandular epithelial cells (Fig. 15.75). Malignant cells vary in the degree they maintain the normal glandular architecture of the magnum and amount of acidophilic secretory granules within their cytoplasm. Implants of acinar tissue are generally well-encapsulated (Fig. 15.76). In some cases cells are agranular and grow in solid sheets. However cytologic differences are not reflective of tumor invasiveness, since implants may be found that are composed of well-differentiated cells. The ultrastructural details of these tumors have been described (80).

Adenocarcinomas of the magnum are extremely malignant. Even when the primary tumor is quite small it may penetrate through the muscularis and spread through the abdominal cavity via tunnels between the celomic membranes to implant on the intestinal serosa, especially the pancreas and duodenum, because they lie deep in the ventral abdominal cavity (84). The muscularis underlying implants on the oviductal or intestinal serosa becomes hyperplastic and hypertrophied. Implants on the intestinal serosa are generally composed of small islands or acini of fairly anaplastic tumor cells encased in dense fibrous tissue (Fig. 15.77).

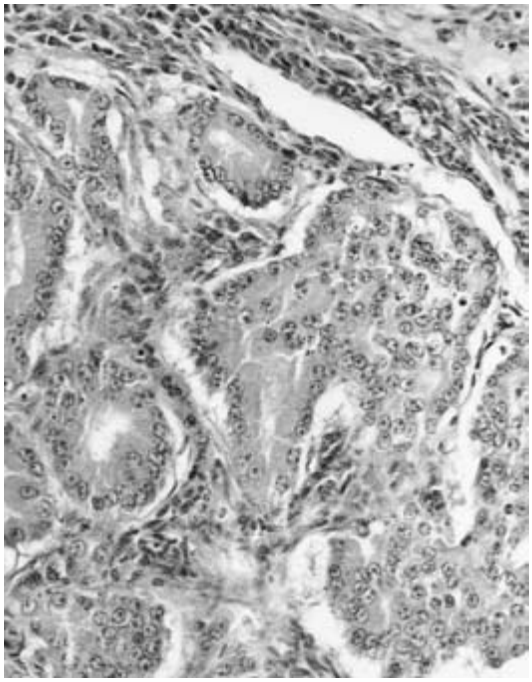


15.74. Dysplastic adenomatous focus in a fold in the magnum showing clear demarcation from surrounding normal glands. The columnar epithelial cells are densely packed and oriented concentrically. H & E, ×175. (Courtesy of *Avian Pathology*)

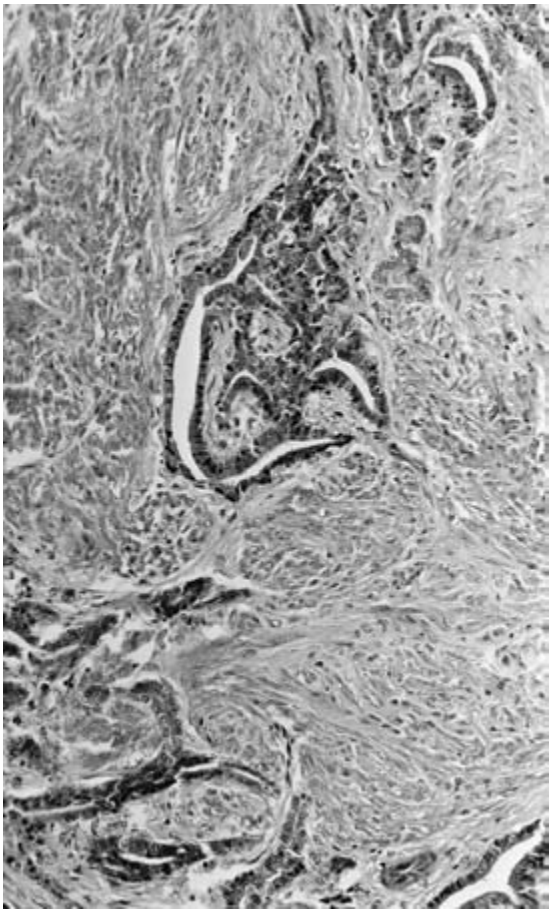


15.75. Magnal adenocarcinoma showing the well-defined margin between normal secretory tissue with cellular cytoplasm containing eosinophilic granules of ovalbumin (above) and very lightly granular tumor cells (below). H & E, ×600.

These are similar, both grossly and histologically, to those produced by ovarian adenocarcinomas, and the ovary itself is a frequent site of implantation. Sometimes metastases may be found quite deep in the ovary. Implants on the oviduct serosa frequently



15.76. Implant of magnal adenocarcinoma deep in the ovary showing capsule around adenocarcinomatous cells. Despite the apparent aggressiveness of this tumor, mitotic figures are not prominent. H & E, ×200.



15.77. Compacted acini lined by cuboidal epithelium surrounded by dense drifts of fibrous tissue in this cirrhotic implant in duodenal serosa of a magnal adenocarcinoma. H & E, ×175.

lack intense cirrhosis (Fig. 15.78). Metastasis to the lungs and other viscera occurs via hematogenous emboli (84).

Immunohistochemical studies showed that these tumor cells contained ovalbumin (72) and retained their receptors for estrogen and progesterone (4). Adenocarcinomas of the magnum were estrogen responsive; their growth was maintained by potent estrogens and suppressed by anti-estrogens (3). Oviductal tumors similar to those found in chickens also have been described in turkeys (14) and metastatic abdominal adenocarcinomas, probably of oviductal origin, have been reported from many other avian species (119).

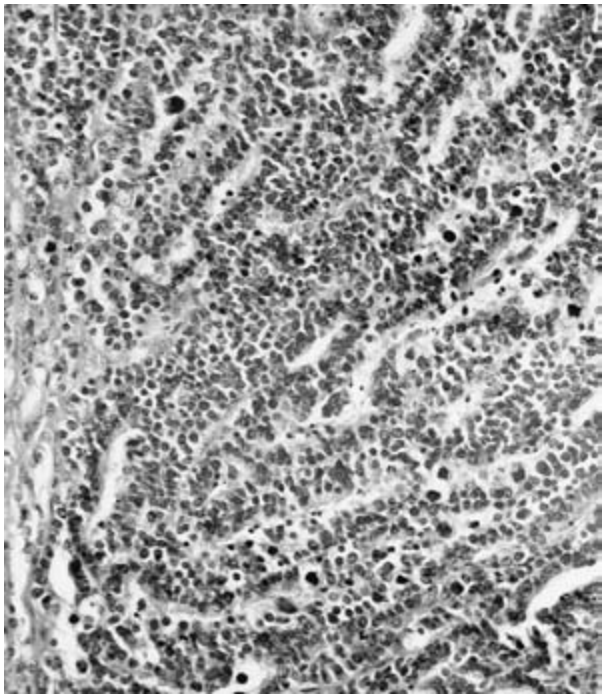
Testis

Teratoma

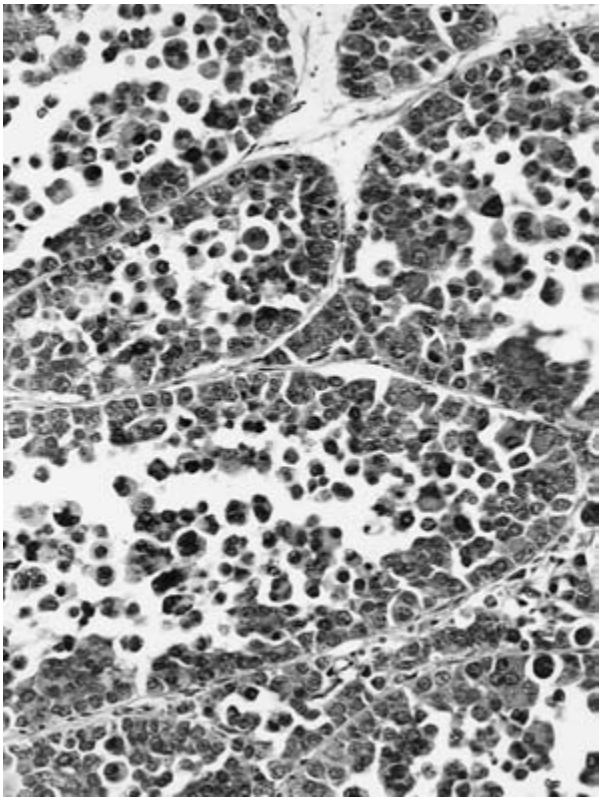
Teratomas are tumors containing multiple cell types arising from more than one embryonic layer. Involvement of the testes appears to be more common than that of the ovary (25, 76), despite more hens than cockerels being kept to sexual maturity. Teratomas also have been found in a number of other sites including the ovary, kidney, adrenal gland, spinal cord, pineal body, and eye (26, 66). They are generally round, yellow to white, encapsulated firm masses that sometimes contain cysts. Several types of teratomas are recognized. One type is composed of bone, cartilage, smooth muscle, nerves, fat, and/or melanocytes. In other types cysts are lined with columnar ciliated epithelium which, along with cartilage and smooth muscle, may form tracheal ring-like structures; additional structures and epithelial pearls formed by squamous



15.78. Magnal adenocarcinoma implanted on the serosa of the isthmus is surrounded by little fibrous tissue. The dilated acinar lumina are lined by cuboidal epithelium. H & E, ×200.



15.79. Sertoli cell tumor in a quail. The tubule-like structures are lined by cells 2 layers deep. H & E, ×360.



15.80. Seminoma in a duck. Lobules of pleiomorphic polyhedral cells with finely granular cytoplasm; some multinucleated cells. Delicate stroma. H & E, ×180.

epithelium may also be seen. Another type of teratoma presents as a sac dilated with fluid and containing fully formed feathers (26). Teratomas that are attached to the spinal column in the lumbar area resemble dermoid cysts of mammals except for the formation of feathers rather than hair. Histologically the sac wall is lined by thin keratinized epithelium and fully formed feather follicles; there are erector pili muscles and nerves in the surrounding tissue. Spontaneous teratomas have been reported in waterfowl (18, 123) and may be induced experimentally by injection of metallic ions into the testes of young adult cockerels (69).

Sertoli Cell Tumor

Sertoli cell tumors have been described in the testes of Japanese quail (62) and budgerigars (119), but they appear to be rare in chickens (25). Grossly they appear as firm, nodular masses with varying degrees of necrosis, hemorrhage, and cyst formation. Histologically well-defined tumors are characterized by Sertoli-like epithelial cells with a large dense basally situated nucleus and basophilic cytoplasm, arranged in a palisading manner around the central lumina of tubules (Fig. 15.79). In other cases the tumor cells are arranged as lobules and sheets separated by delicate stromata. Mitotic figures are common. The number of interstitial cells between these tubules and islands is variable, and in some cases such cells may be vacuolated. In mammals Sertoli cell tumors may be associated with estrogen production and feminization. A case of feminization has been described in an incompletely surgically castrated cockerel wherein a Sertoli cell tumor arose from the gonadal remnants (133); and several examples of demasculinization have been reported in budgerigars with Sertoli cell tumors (12). Feminization in avian species requires demasculinization by female hormones but hormonal studies in affected poultry have not been reported.

Seminoma

Seminomas are large unilateral tumors with a well-defined capsule and histologically are composed of loose sheets or compact cords interspersed with a delicate stroma (Fig. 15.80). The cells are large and round, containing a round-to-oval nucleus with prominent nucleoli (24). Occasional syncytia are noted and mitotic figures are numerous. Seminomas have also been reported in ducks, quail, and budgerigars (12, 53, 119).

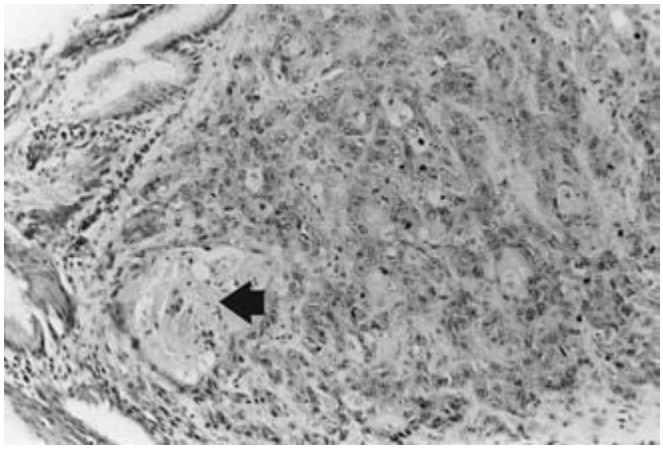
Leydig Cell Tumors

Neoplastic Leydig cells may be a component of a seminoma. Leydig cell tumors are composed of large polygonal cells with eccentrically placed vesicular nuclei and granular acidophilic, sometimes vacuolated, cytoplasm arranged in irregular acini.

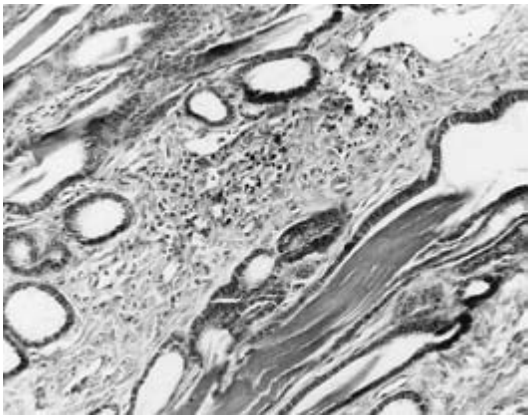
Digestive System

Alimentary Tract

Pharyngeal and esophageal squamous cell carcinomas in chickens have been described (1, 30, 122). A high incidence of this



15.81. Oro-pharyngeal squamous cell carcinoma in an adult non-commercial chicken. Cords and islands of epithelial cells with some central keratin pearls (arrow). Mucosal gland is shown at top left. H & E, ×200.



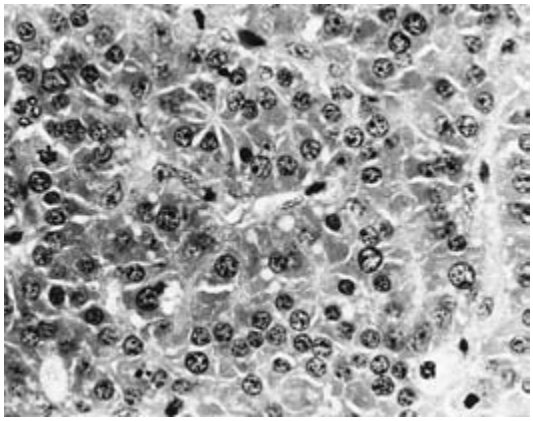
15.82. Adenocarcinoma of gizzard with growth of darkly staining cuboidal tumor cells downward into muscularis. The keratinous product of these cells is shown at lower right. H & E, ×200. (K. Langheinrich)

tumor has been reported in chickens from northern China, and humans in the same area also have a high incidence of esophageal carcinoma (27, 114, 131). In southern China a relatively high incidence of a homogenous group of squamous cell-derived carcinomas of the nose, mouth and pharynx of humans is associated with Epstein-Barr virus (109), but it has been proposed that there may also be a significant contribution of genetic predisposition, and environmental or ingested chemicals. Perhaps some similar factors are operative in the oro-pharyngeal squamous cell carcinoma of chickens. These tumors were composed of cords or islands of epithelial cells in the lamina propria and deeper tissues, with formation of some central keratin pearls. Superficial ulceration and infection was common (Fig. 15.81).

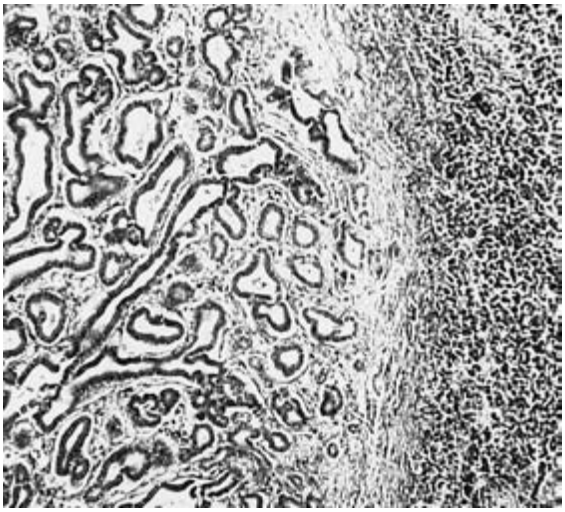
Papillomatous-like growths in the esophagus and crop of chickens have been reported, but their etiology and pathogenesis are not known (106). Internal papillomatous disease of psittacines, particularly macaws and Amazon parrots, often affects the cloaca. The lesions vary from irregular hyperplastic epithelial cells supported on a fibrovascular stalk extending from the lamina propria to poorly differentiated cords of adencarcinomatous tissue. A study in neotropical parrots revealed all cloacal papillomatoid lesions contained psittacine herpesvirus DNA but it was absent in the adjacent non-affected tissues: psittacine herpesvirus genotypes 1, 2 and 3 were identified in these lesions (139). Bile duct and pancreatic adenocarcinomas also tend to occur more frequently in psittacines with mucosal papillomas and similarly psittacine herpesvirus DNA was reported in these (138). No evidence was found of involvement of papillomaviruses in these cases. Herpes-like virus particles were observed in a cloacal papilloma of a conure (61). Cutaneous papillomatoid-like lesions in fish and marine reptiles are also associated with herpesvirus infections. Papillomatous nodules on the mucosa, skin and commisures of the beak associated with papillomaviruses occur in canaries (40), finches, African gray parrots and Cuban Amazon parrots (139). In such cases basophilic intranuclear inclusions may be noted in the hyperkeratotic proliferative epithelium. Small polypoid-like nodules are noted from time to time on the conjunctival mucosa of chickens and other birds, particularly towards the muco-cutaneous junction: they often overlie a prominent capillary bed and the epithelium is moderately hyperplastic and hypertrophied, and folded into small papillomatoid or polypoid nodules. The cases in poultry have not been studied in detail.

There have been several reports of adenomas of the crop, esophagus, proventriculus, and gizzard of birds (9, 26, 92, 119, 121). Adenocarcinoma of the gizzard has been observed in U.K. and U.S.A. broiler chickens (Fig. 15.82) (26), an adult hen (122) and other avian species (31). Guerin (66) described five epithelial tumors of the small intestine and one of the ileocecal junction, and cited several other reports of intestinal carcinomas in chickens. In these cases gross examination revealed papillary projections of tumor tissue into the lumen of the affected organ, sometimes with penetration of the muscularis by invading epithelial tissue, which formed acinar or cystic structures containing mucin. Solitary nodular adenocarcinomas of the intestinal mucosa of chickens also have been described (121, 147). Campbell (25) noted that reports of intestinal adenocarcinoma in the chicken should be viewed with caution, as such tumors can be difficult to differentiate from metastatic abdominal adenocarcinomas that arise from the reproductive tract and frequently implant on the intestinal serosa and infiltrate through the muscularis into the underlying mucosa and then proliferate there as a solid nodule.

Leiomyomas may be found in the muscularis of the gizzard or intestines (see "Musculoskeletal System"). In pheasants and peafowl pseudoneoplastic nodules of proliferating fibrous tissue in the cecal wall may be induced by larval stages of *Heterakis isolonche* (65). Enterogenous cysts derived from gastrointestinal tract mucosa have been described in chickens (87).



15.83. Hepatoma composed of large eosinophilic neoplastic cells, some in mitosis, forming irregular plates. H & E, ×600.



15.84. Cholangioma composed of dilated ducts in a loose fibrocytic stroma. H & E, ×75.

Liver

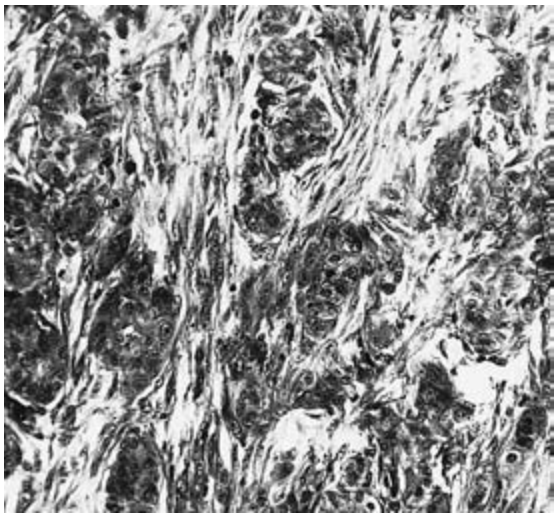
Hepatocellular Tumors

Spontaneous neoplasms of hepatocytes appear to be rare in chickens, and only occasional reports of either benign trabecular hepatocellular adenomas or anaplastic carcinomas have been published (25, 32, 106, 121). Typical hepatocellular adenomas grow in the hepatic parenchyma as a large, circumscribed, soft, yellow-gray mass. Histologically they are composed of polygonal eosinophilic cells about double the size of normal hepatocytes, forming thick, irregular cords lacking normal hepatic triad structures (Fig. 15.83). Mitotic figures are rare. Hepatocellular carcinomas are often multifocal nodules composed of sheets of basophilic neoplastic cells somewhat smaller than those in hepatomas and with numerous mitoses (115); metastasis to the lung may occur. Such tumors are similar to hepatocellular carcinomas induced with transforming avian retroviruses, most notably avian leukosis virus strain MC29 (13). A chicken hepatocellular carcinoma cell line was derived from a typical tumor induced by long-term parenteral treatment with diethyl-nitrosamine (85).

Hepatic tumors also have been reported in ducks (23) and appear to be inducible in this species with aflatoxin (29). Additionally hepatocellular carcinomas in ducks have been associated with duck hepatitis B virus (163). The incidence of hepatic tumors in Chinese ducks is high, ranging from 2 to 15%, with hepatocellular carcinomas being most common (95). The role of genetics, age, diet, and other environmental or virologic factors is not known. Hepatocellular tumors have been described in a variety of other avian species (119, 149).

Cholangiocellular Tumor

Tumors of the biliary system are not common in chickens. They are generally firm, demarcated from normal hepatic parenchyma, and yellow-gray in color. The histology varies according to the degree of malignancy. Cholangiomas are composed of clearly recognizable but enlarged tubules resembling distorted bile ducts, interspersed with fibrous connective tissue (26, 52, 121) (Fig. 15.84). In cholangiocarcinomas duct formation is irregular



15.85. Cholangiocarcinoma composed of small clusters of epithelial cells in a fibroblastic stroma. H & E, ×190.

and the connective tissue is fibroblastic (Fig. 15.85). Infiltration between hepatic cords is aggressive. These tumors of bile ducts need to be differentiated from chronic hepatotoxin induced bile duct proliferation that is often accompanied by fibrosis and distorsion of hepatic architecture. Cholangiocellular tumors have been described in pigeons (153) and other birds (113, 149).

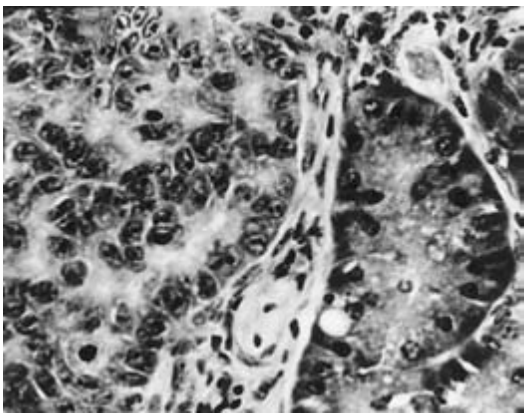
Pancreas

Adenocarcinoma

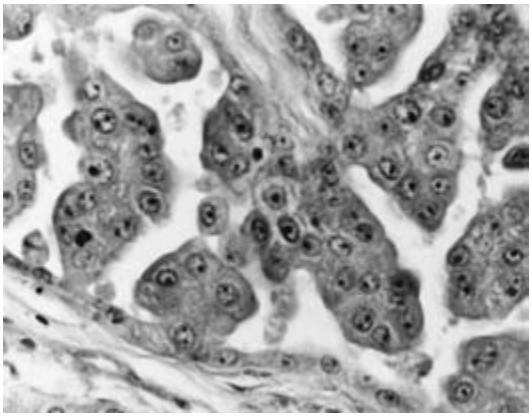
Tumors of the pancreas are difficult to differentiate from metastatic abdominal adenocarcinomas derived from the ovary or oviduct, that frequently implant on the serosa of duodenal loop and pancreas and then invade the pancreas. There can be absolute



15.86. Pancreatic adenocarcinoma, probably of ductule cell origin, composed of columnar cells forming tubular structures among a few remnant acinar cells (arrow). ×160.



15.87. Pancreatic acinar cell adenocarcinoma with normal exocrine tissue (right) and agranular neoplastic cells (left). H & E, ×600.



15.88. Mesothelioma with prominent neoplastic epithelial cells supported on a delicate stalk. H & E, ×600.

certainty of a pancreatic primary tumor only in absence of ovarian or oviductal involvement, as in the case of the male Guinea fowl reported by Okoye and Ilochi (105). Extensive metastatic implants to the serosa of the duodenum and proventriculus, and the hepatic capsule can occur, but the ovary is not involved. Most pancreatic adenocarcinomas probably originate from ductal epithelium, not acini. They are composed of tubular structures lined by columnar epithelial cells with lightly basophilic cytoplasm (Fig. 15.86). The basal nuclei are round to oval, and mitotic figures are prevalent. In one case observed by Fredrickson and Helmboldt (52), the tumor appeared to arise from exocrine tissue, rather than ducts, because a clearly defined transitional zone between normal acini and tumor tissue could be distinguished. The large neoplastic cells had extremely vesicular, round nuclei, and cytoplasm contained a variable number of the same deeply eosinophilic granules typical of normal acinar cells (Fig. 15.87).

Peritoneum

Mesothelioma

Mesotheliomas have been reported in chickens (66, 106), ducks (94), a hawk (35), and ratites (119). In this tumor both the surface lining the mesothelial cell and the underlying connective tissue are involved. One case in a S.P.F. hen was described by Fredrickson and Helmboldt (52); the abdominal cavity contained about 200 mL of milky fluid and the serosal surfaces were covered by glistening, gray cystic structures. Histologically these were composed peritoneal cells supported by thick connective tissue stromata that formed the walls of the cysts and are projected into these papillary (Fig. 15.88). Mitotic figures were rare, but tumor growth was extensive.

Urinary System

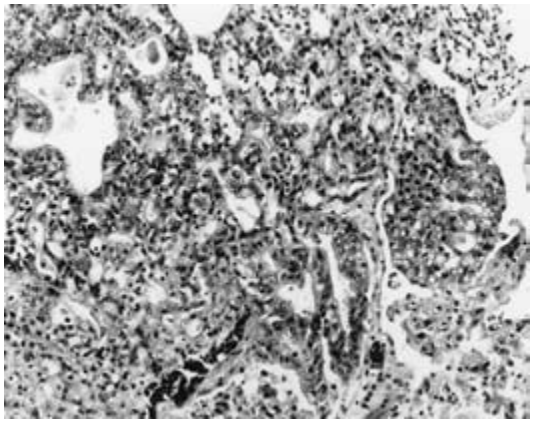
Renal adenocarcinomas (nephromas) and nephroblastomas may occur as spontaneous neoplasms in chickens, but they are inducible with avian leukosis virus and are described elsewhere in this book (see "Leukosis/Sarcoma Group"). Renal adenocarcinomas are common in budgerigars (103), but their etiology is not known.

Respiratory System

Infra-orbital Sinus

Adenoma

A hen and a cockerel from unrelated noncommercial free-ranging poultry flocks were presented with single pea-sized cysts (single in the hen, two in the cockerel) underneath the eye. The cysts were well-encapsulated, extended into the infra-orbital sinus and exuded mucinous material when cut. They were composed of cuboidal epithelial cells arranged as acini with fluid filled lumina: mitotic figures were rare (122).



15.89. Adenocarcinoma of lung composed of papillary growth of epithelial cells that have replaced most of the normal lung. H & E, ×200.

Lung

Adenocarcinoma

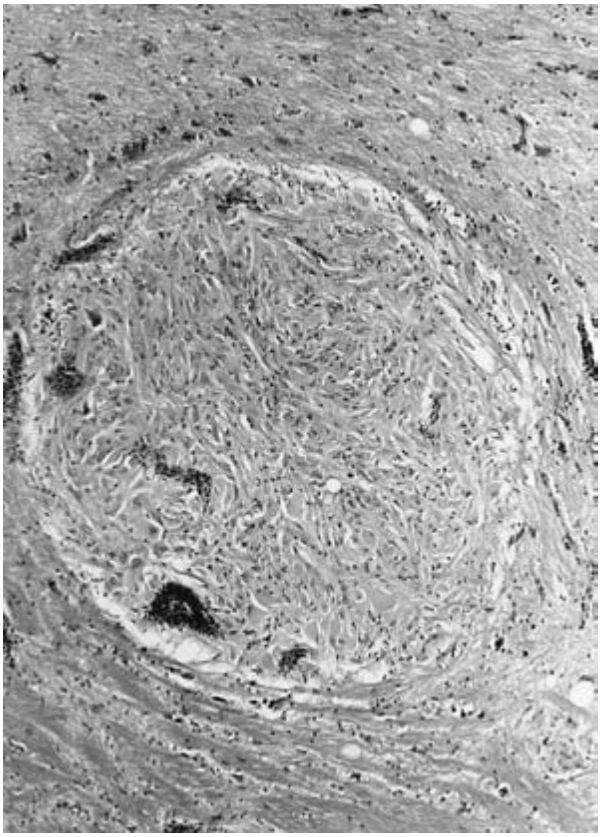
Tumors of the respiratory tract of poultry are rare, with Campbell describing only three cases of pulmonary adenocarcinomas in domestic fowls (25). Ducks appear to be more prone than other avian species to pulmonary tumors, as there are several reports of naturally occurring pulmonary adenocarcinomas in ducks (96, 164) and a variety of lung tumors were induced in Pekin ducks given a chemical carcinogen intratracheally (124). Stewart (137) described 20 cases of pulmonary adenocarcinomas or adenomatosis in birds, including 11 in ducks. Most of these cases appeared to originate from the bronchial epithelium. Adenocarcinomas of magnum of the oviduct and other tumors (rhabdomyosarcomas, myxosarcomas, and fibrosarcomas) can metastasize to the lungs of domestic fowl (121), and such tumors need to be differentiated from adenocarcinomas arising from within the lungs. The case described by Fredrickson and Helmboldt (52) clearly arose multifocally from parabronchi and resembled papillary adenocarcinomas as described by others (6, 137). In that case cuboidal epithelium formed distorted bronchial-like tissue often containing eosinophilic material (Fig. 15.89). Widespread and distant metastases in the thorax and abdomen attested to the extreme malignancy of this tumor.

Nervous System

Central Nervous System

Astrocytoma

Sporadic cases of astrocytoma have been described (15, 82, 83, 121). Wight and Duff (158) investigated a small epizootic affecting 20 birds out of a flock of 1000, of which 13 were examined histologically. Adult birds are usually affected, and all five cases examined by the author (121) were in aged noncommercial hens. Clinical signs include transitory torticollis, retropulsion, and incoordination. Astrocytomas are frequently multiple, unencapsulated small nodules, usually located in the base of the cerebellum or underlying rostral brain stem. Although each tumor is small, usually no larger than 5 mm in diameter, they may be seen without difficulty, especially in fixed tissues, as sharply delineated whitish masses. There is frequently a marked perivascular reaction of lymphocytes bordering the tumor, but no hemorrhage, giant cells, or areas of pressure necrosis (Fig. 15.90). The neoplastic cells vary considerably in morphology, but are mainly polygonal with extended cytoplasmic fibrillar processes (Fig. 15.91): these processes can be enhanced with phosphotungstic acid hematoxylin stain. Astrocytomas need to be differentiated from reactive gliosis induced by migrating parasites.

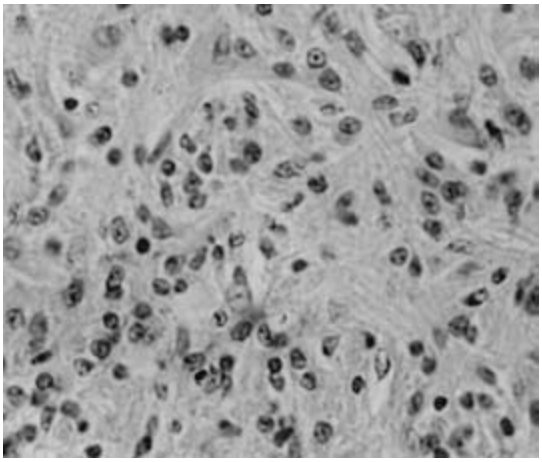


15.90. One of several clearly demarcated but unencapsulated astrocytomas composed of fibrillar astrocytes, in anterior brain stem. There is a significant lymphocytic infiltrate around the blood vessels within the tumor and the adjacent tissue. H & E, ×100. (Courtesy of *Avian Pathology*)

Wight and Campbell (157) reported finding an ependymoma and two meningiomas in chickens. The former was a growth in the lateral ventricles of palisaded or rosette-forming, vacuolated cells, while the meningiomas were of the angioblastic variety composed of vascular sinuses lined with plump endothelial cells associated with a dense network of reticulin. Several ependymomas and a choroid plexus papilloma were identified in the brains of budgerigars, and found to be S-100-protein positive (140).

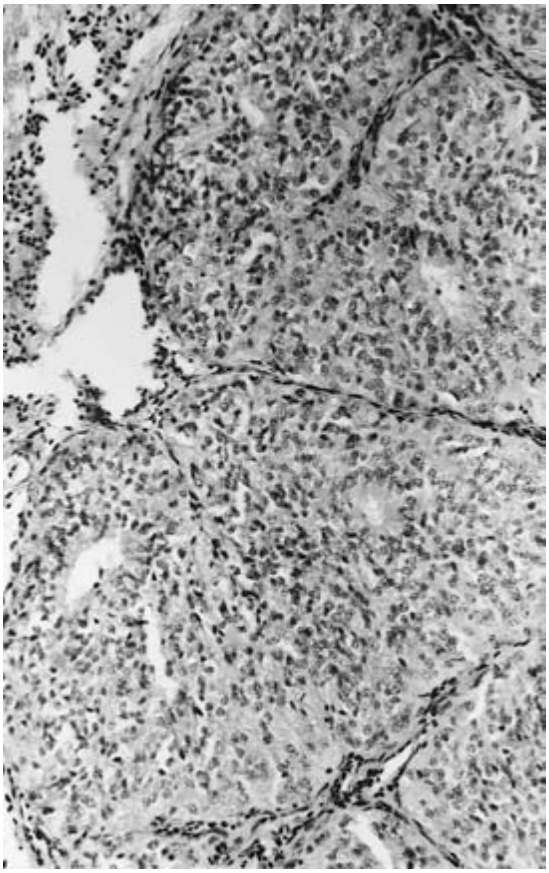
Pineal Body Tumor

There are several reports of avian pineal body tumors (25, 119, 121, 144, 160). Swayne *et al.* (144) used several criteria to differ-



15.91. Astrocytoma composed of uniform astrocytes with extended cytoplasmic processes. H & E, ×190.

entiate pineal body tumors from hyperplasia: in tumors, the pineal body was greatly enlarged, impinged on the adjacent cerebellum, and mitotic figures were present in the epithelial cells. There may be clinical signs such as fine tremors and head pressing, and a large encapsulated mass can be found between the cerebellum and the cerebrum, often impinging onto or even embedded into the cerebellum. The tumor is composed of lobules of epithelial cells comprising low columnar cells arranged in a palisading manner around a lumen, and there are abundant parafollicular cells with dense nuclei, separated by fine trabeculae (Fig. 15.92).



15.92. Lobules of a pineal body tumor separated by fine trabeculae. Palisaded low columnar epithelial cells with large vesicular nuclei are arranged around a small lumen, and these cells are surrounded by smaller parafollicular cells. H & E, ×200. (*Avian Pathology*)

Peripheral Nerves

Schwannoma

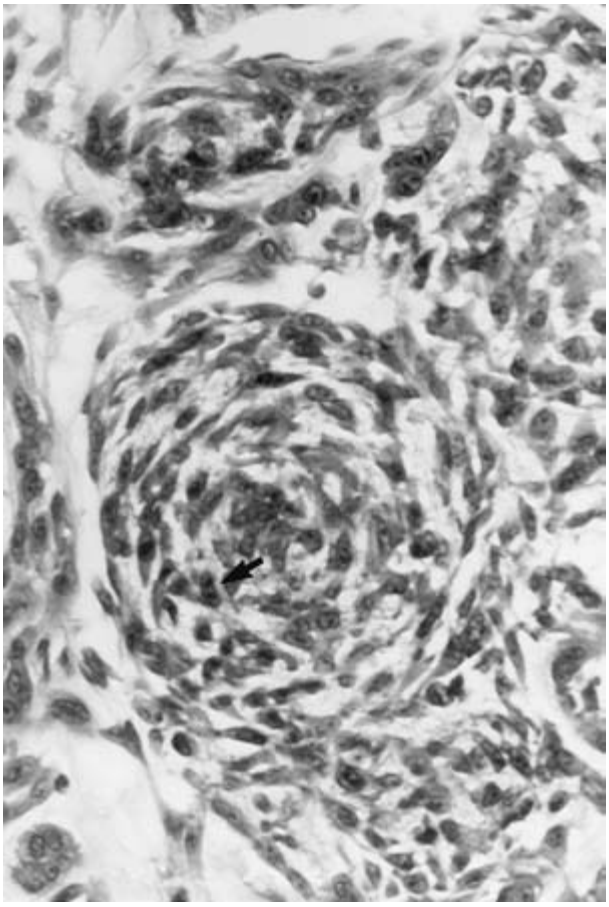
Tumors of the Schwann cells or perineural cells of peripheral nerves are preferably called Schwannomas, although often they are designated as neurofibromas, neurilemmomas, or neurogenic sarcomas; differentiation between these is difficult and they are best considered together. Campbell and Appleby (26) reported 39 tumors of the nerve sheath in broiler chickens and reviewed 17 other cases, of which some were in adult birds. They are generally benign localized tumors forming white nodular or fusiform growths, most often in the region of the dorsal root ganglia. The tumor cells are spindle shaped with a small central nucleus, and usually they form concentric whorls reminiscent of nerve sheaths (Fig. 15.93). Multiple nodular tumors have been reported (2), including a congenital case (26). Tumors described as neurilemmomas had cells arranged in a palisading manner with occasional structures resembling Wagner-Meissner tactile corpuscles (25). Tumors resembling Schwannomas should only be so designated if the nerve of origin is identified or they can be confirmed as being of Schwann cell origin (19); otherwise they are best described as fibrosarcomas to avoid confusion. Some cases of Schwannomas resemble hemangiopericytomas.

Neuromas

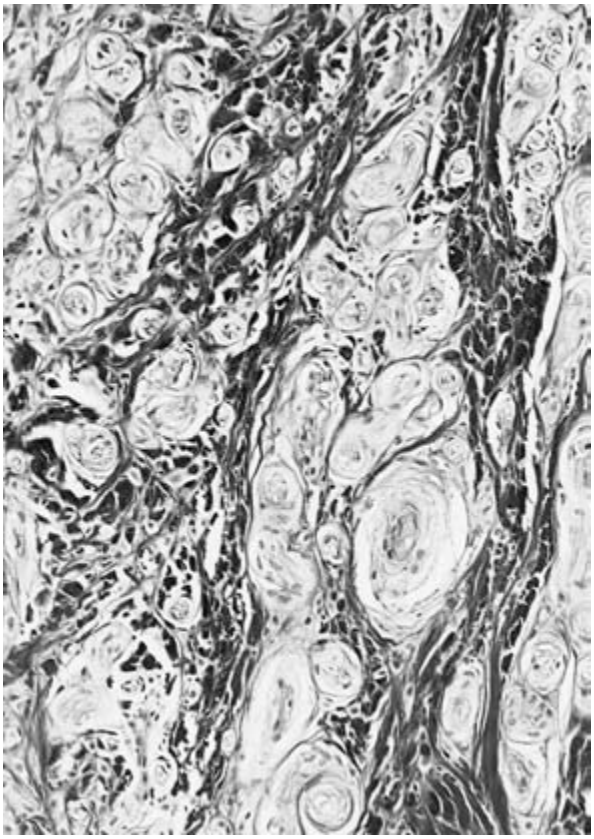
Amputation of the tip of the beak of juvenile chickens, turkeys and other poultry, and partial amputation of the hallux of male broiler breeders, may result in the formation of neuromas (54, 55). In neuromas there is a nodular or diffuse thickening due to proliferating nerve bundles within a dense collagenous matrix. The pathogenesis is similar to post-traumatic neuromas of domestic mammals and humans where a regenerating nerve stump encounters an obstruction such as dense fibroblastic scar tissue and cannot re-innervate normal dermal tissue so it proliferates as a tangled mass of axons, Schwann cells and associated connective tissue (Fig. 15.94). The axons can be identified by staining with Holme's silver method, but they are thinly myelinated. Technically these are an abnormal regeneration rather than neoplasia. Partial beak amputation of the beak in young chicks results in loose dermal scar tissue and that is probably the reason they are not prone to neuroma formation (38). Compared with chickens, partial beak amputation of turkeys also appeared to be associated with less dense scar tissue and neuromas did not form (56).

Melanoma

Melanocytes are derived from the neural crest. Thus, melanomas are considered under tumors of neural tissue. Melanosis is common in many avian species, and breeds of chickens such as silkies



15.93. Schwannoma of the sciatic plexus showing concentric whorling pattern of spindle-shaped cells with central nuclei. Note the mitotic figure (arrow). H & E, ×400.



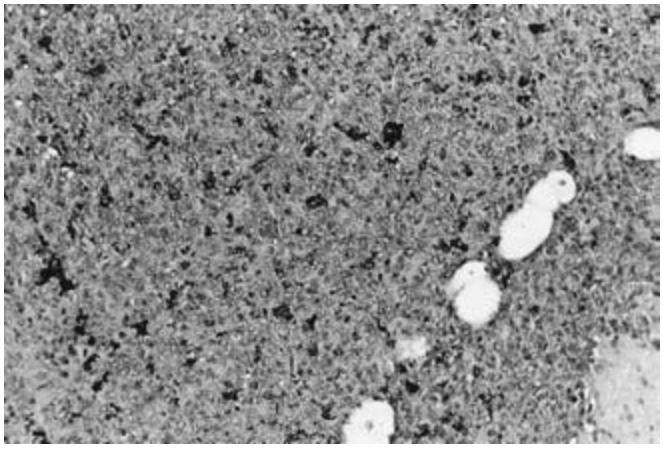
15.94. Post-traumatic neuroma in beak tip showing dense collagenous scar tissue and multiple whorls of nervous tissue composed of poorly myelinated axons, Schwann cells, and associated connective tissue. Martius scarlet blue, ×360. (C. J. Randall)

have numerous foci of melanocytes especially in gonads, peritoneum, perineurium, and periosteum. Campbell (25) reviewed a number of cases of melanoma and noted that malignant forms may arise in the ovary and metastasize throughout the abdominal cavity. The eye also can be a primary site of melanomas (42). Malignant melanomas have been observed in ducks (39, 58, 119), a cormorant (89), and budgerigars (128). Melanomas with varying degrees of melaninization occur in the subcutis of racing pigeons (117, 122), and multifocal melanomas have been observed, although rarely, in chickens (121): in such cases mitotic figures are infrequent. As in mammalian species the morphology of the cells in avian melanomas vary from fusiform to elongate and pleiomorphic, and they may be arranged as discrete foci, as infiltrates into the surrounding tissue, or as more superficial small islands of closely packed melanocytes reminiscent of epithelial tissue (Fig. 15.95). Excess melanin can be bleached from sections using various techniques, such as with H_2O_2, to reveal occasional multinucleated cells and numerous mitoses. Melanin granules may be sparse, and in such cases, the brown pigment can be enhanced with ammoniacal silver techniques such as modified Masson-Fontana stain. The D.O.P.A. (L-3,4-dihydroxyphenylalanine) test can be used on unfixed relatively amelanotic melanomas to detect the presence of tyrosinase, a necessary enzyme in the biosynthesis of melanin and other substances. The S-100-protein family contains a large number of related calcium binding proteins found in mammalian dermal melanocytes and other cells such as nervous, lymphoid and endothelial tissues. Therefore S-100-protein immunoreactivity is considered a useful marker of mammalian melanomas, however, limited studies have revealed that the range of tissues which reacted in avian species, represented by the duck, was somewhat different to that in mammalian species (142). A melanoma in a cormorant was S-100-protein negative but the adjacent peripheral nerve bundles were positive (89).

Special Senses

Eyes

Ocular tumors besides lymphomas and myelomas are rare in birds. The avian iris muscle is striated and intra-orbital rhabdomyosarcomas were detected in juvenile and subadult chickens (42). Cole (33) described a retinoblastoma. Melanomas and teratomas may also involve the eye. Osteosarcomas may originate in the orbit, but these need to be differentiated from intra-ocular ossification, a not unusual sequellae to progressive retinal degener-



15.95. Subcutaneous melanoma of the wing of a pigeon. There is melanin pigment in many polyhedral cells. Mitotic figures are rare. The cells are densely packed, clearly demarcated from the surrounding tissue, and penetrate between muscle fibers and adipocytes as shown at lower right. H & E, ×200.

ation (86) or chronic intra-ocular infections such as toxoplasmosis (148).

Endocrine System

Thymus

A few thymomas, as distinct from thymic lymphomas, have been reported in chickens (26, 47, 66, 106), a duck (162), a budgerigar (165), and a Java sparrow (99). They are characterized by replacement of the normal thymic architecture with sheets of large polyhedral epithelial cells interspersed with variable numbers of lymphoid cells. No structural pattern is obvious, although there may be poorly defined lobules. The vesicular nucleus and abundant pale-staining cytoplasm of the epithelial cells contrast with normal lymphocyte morphology. The borders of the neoplastic cells are indistinct but may show squamous differentiation. The epithelial origin of these cells was confirmed by immunostaining for cytokeratin (99). Aggregates of cells resembling Hassall's corpuscles are sometimes mixed among the tumor cells.

Pituitary Gland

Pituitary adenomas have been reported in budgerigars (10, 129, 140), and although frequently cited as common, the true incidence in this species is not known: the 50 cases described by Schlumberger were solicited from throughout U.S.A. by targeted advertisements (129). Campbell (25) reported two pituitary adenomas in domestic fowls. None were seen by Fredrickson and Helmboldt, although pituitary glands were examined in several hundred aged hens (52), nor were any seen in the brains of many chickens or other birds showing neurologic signs, which were examined histologically by the author (74, 120, 122).

Adrenal Gland

The avian adrenal gland has an intermingling of inter-renal (cortical) and enterochromaffin (medullary) tissues and tumors can arise from either. Campbell and Appleby (26) recorded a single case of an adenoma they considered was most likely derived from the adrenal gland, and an adenoma involving the adrenal gland was seen in an adult S.P.F. hen (121). These adrenal adenomas were composed of well-differentiated cells with abundant eosinophilic cytoplasm, but there were no particular histologic characteristics that would allow definitive confirmation that these tumors were derived from adrenal tissue. Similar tumors have been described in other avian species (119). An adrenal tumor in a budgerigar was studied ultrastructurally, and cytoplasmic granules consistent with those in adrenal medullary tissue were observed thus meriting the tumor's description as a pheochromocytoma (71).

Thyroid and Parathyroid Glands

Guerin (66) described an adenoma arising in the parathyroid gland of a chicken and pointed out that only one other case of parathyroid carcinoma had been reported in chickens. Tumors of the thyroid gland in poultry appear to be extremely rare (25, 106). Naturally occurring goiters have been reported in chickens (66), and were induced in chickens and quail by feeding rapeseed meals containing goitrogens (159). Goiters also occur in budgerigars, probably as a consequence of low iodine intake (16, 119). In budgerigars neoplasia of the thyroid gland may be difficult to differentiate from hyperplasia and dysplasia associated with goiter; however, in the thyroid adenomas reported by the author (119), there were discrete areas of neoplastic adenomatous tissue. A mixed cell tumor of the thyroid gland was composed of adenomatous tissue and islands of proliferating chondrocytes and fibroblasts.

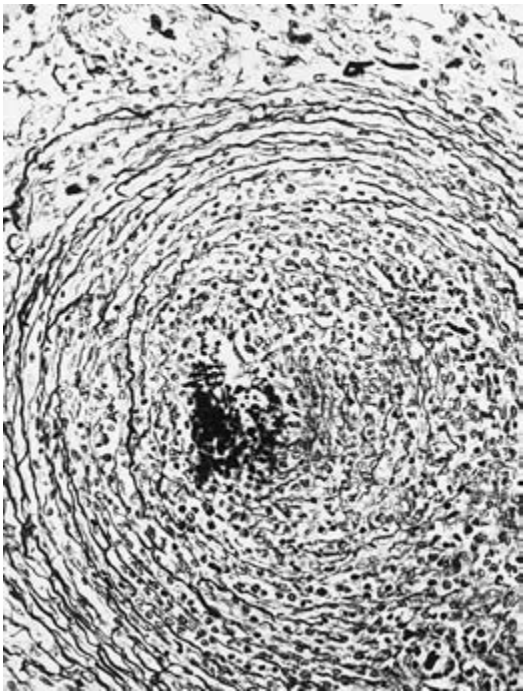
Integument

Subcutis

Soft tissue tumors such as fibromas, fibrosarcomas, myxomas, and myxosarcomas are encountered in the subcutis of chickens and may be induced by avian leukosis virus (see "Leukosis/Sarcoma Group"). Occasional cases are reported in S.P.F. poultry and other species. Myxomas and fibromas were observed on the rostral extremity of the upper trimmed beaks of hens (121). The etiology of these was not determined but it was postulated that they may have resulted from retrovirus-induced transformation stimulated by local injury.

Hemangiopericytoma

A few hemangiopericytomas in the subcutaneous tissue of chickens have been described (52, 134). All of these were benign tumors occurring as subcutaneous nodules of variable size, usually in the cervical region. The nodules were dense, white, well-delineated, and firmly embedded in the subcutis. The histologic appearance was of uniform spindle-shaped cells possessing a fusiform nucleus with diffuse chromatin, and abundant cytoplasm but indistinct cell borders. They were arranged in a concentric manner around central blood vessels, and the intervening reticulin fibers could be readily demonstrated by a silver stain (Fig. 15.96).



15.96. Hemangiopericytoma with concentric rings of pericytes clearly defined. Silver, ×90.

Lipoma and Liposarcoma

Subcutaneous lipomas in mammals often arise at sites of trauma, but they are not common in chickens (25). However subcutaneous and intra-abdominal lipomas are frequently encountered in other avian species, especially psittacines (90, 119). They are generally encapsulated, benign, delicately trabeculated tumors with variable degrees of necrosis and hemorrhage. They are composed of mature adipocytes with large cytoplasmic vacuoles and a displaced pale nucleus. Mitotic figures are rare. Malignant liposarcomas of chickens are rare and may be locally invasive or metastasize (101, 121). They may be similar in histologic appearance to fibrosarcomas except for intracytoplasmic fat vacuoles within tumor cells. In other cases the tumor may be composed of obviously immature adipocytes. Multifocal liposarcomas have been described in other avian species (41, 119).

In other avian species as well as in humans and other mammals a variation of lipoma known as myelolipoma or erythrolipoma is reported. This presents as a well-delineated benign mass with foci of myelopoietic and/or erythropoietic elements embedded in adipose tissue corresponding to bone marrow but in an abnormal location. They have been recorded in the subcutaneous tissue of the limbs or liver (91). They need to be differentiated from extramedullary erythropoiesis and/or myelopoiesis.

Cutis

Squamous Cell Carcinoma

The tumor of broiler chickens commonly referred to as dermal squamous cell carcinoma, in some aspects the avian equivalent of a keratoacanthoma, is dealt with elsewhere in this book. A true squamous cell carcinoma is a malignant tumor of keratinocytes and forms irregular masses or cords that proliferate downward and invade the dermis and subcutis. It is characterized by aberrantly located dermal epithelial cells with intercellular bridges resembling the stratum spinosum. There is no cushion of basal cells and the epithelial tumor cells lack orderly maturation, although some keratinization usually is found. These tumors are associated with an intense mononuclear inflammatory cell infiltrate and a reactive fibroplasia. Squamous cell carcinomas are locally invasive, but may be slow to metastasize. There are only a few reports of true dermal squamous cell carcinomas of chickens in the literature and most of these affected the scaly skin of the shanks and lower feet of adults (25, 28, 141). Several cases of squamous cell carcinoma involving the oropharynx of chickens have been reported (see above). In other avian species dermal squamous cell carcinoma may involve the uropygeal gland and these need to be differentiated from impaction, trauma and/or uropygeal adenocarcinomas (130).

Feather Folliculoma

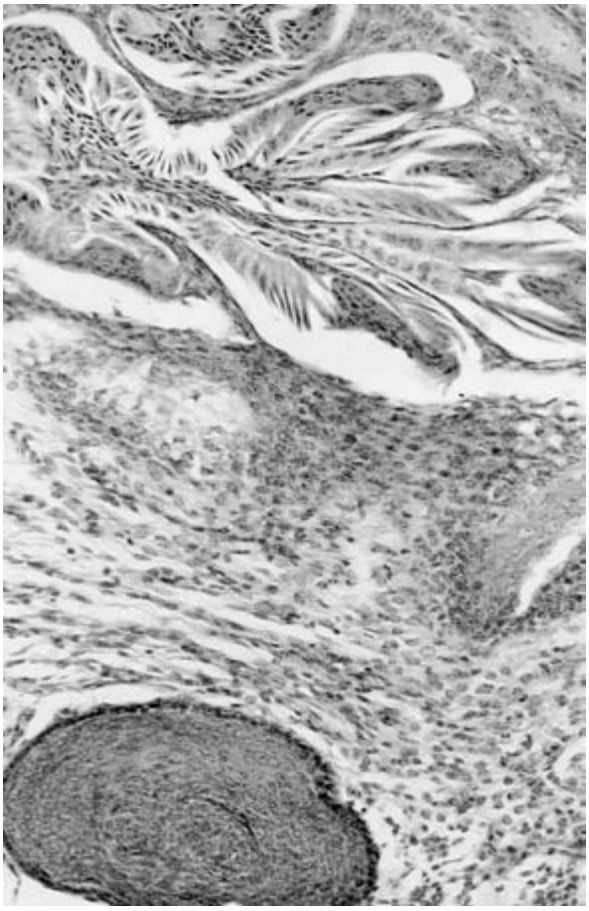
Feather folliculomas are usually multiple cystic structures with central lumina containing keratinized debris and feather remnants. They are lined by cuboidal to squamous epithelial cells with abrupt keratinization, and areas of disorganized feather follicle epithelium. There is usually an intense inflammatory cell infiltration into the surrounding dermis and some fibrosis (Fig. 15.97). Feather folliculomas of chickens (121) and turkeys (36) have been described, and they are common in some caged birds such as Norwich canaries (108).

Intracutaneous Keratinizing Epithelioma

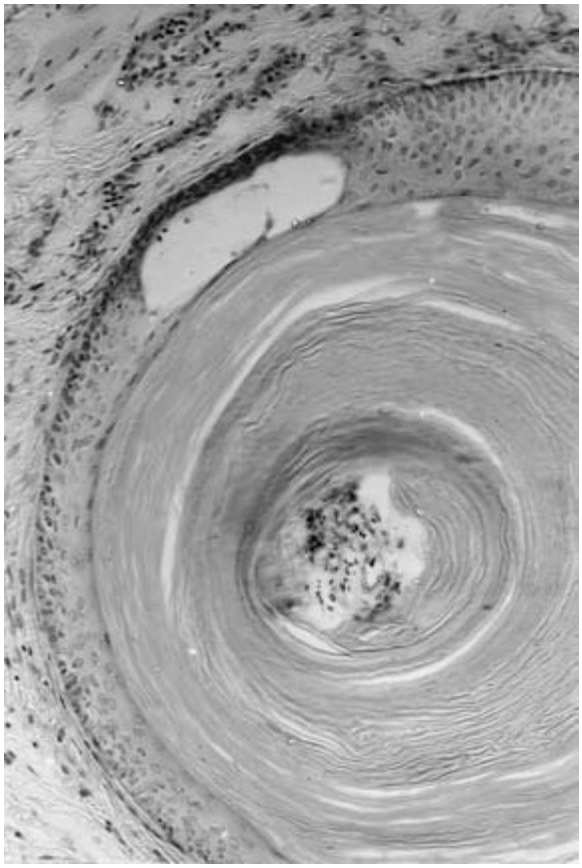
These are a benign cystic tumor of the facial skin of adult chickens. They present as multiple small well-encapsulated nodules with a central craterous pore (121). They are lined by well-developed stratified epithelium consisting of basal cells progressing to maturation with prominent intercellular bridges in the stratum spinosum. The lumina contain lamellated keratin but no feather remnants (Fig. 15.98). They are surrounded by a small amount of fibrous tissue and there is little inflammatory cell reaction unless there is rupture of the wall. These tumors are probably derived from keratogenous cysts and are distinct from both keratoacanthomas of broilers and feather folliculomas.

Other Tumors of the Cutis

Hard, horny, papillomatoid tumors of the scale-producing epithelium of the shanks, with heavily keratinized whorls, are acanthomas (25). Uropygeal adenomas may arise from the preen (uropygeal) gland situated dorsal to the base of the tail in the chicken. Papillomatous-like lesions on the footpads of French ducks were reported but not described in detail, and an association with papillomatous lesions in abattoir workers was postulated (67); their etiology was not determined. Acanthotic and hyperkeratotic lesions found on the footpad of a duck were associated with herpesviruses (161), but the relationship of that to other footpad neoplasms, such as those described elsewhere (see "Chondroma and Chondrosarcoma") is not known.



15.97. Edge of a feather folliculoma showing dysplastic specialized feather-forming epithelium and an adjacent cord of basal cells. The lumen was lined by stratified cuboidal to squamous epithelium with abrupt keratinization and contained keratin and feather remnants. H & E, ×180.



15.98. The lumen of this intracutaneous keratinizing epithelioma contains lamellated keratin. The epithelium shows basal cells aligned on a distinct basal lamina and progression to polyhedral cells with abrupt keratinization. Note the small intraepithelial bulla. H & E, ×190. (Courtesy of *Avian Pathology*)

Xanthomas are yellow subcutaneous nodules sometimes found in gallinaceous and psittacine birds, often overlying lipomas. They are composed of lipid-laden macrophages, giant cells, free cholesterol and fibrous tissue: they are not true tumors. Multiple xanthomas were noted in chickens in the U.S.A. in the 1950s, possibly due to a reaction against feed contaminant metabolites accumulating in subcutaneous fat (126).

Multiple small firm white nodules in the skin of a hen were found to be cutaneous mast cell tumors. The round to oval cells, with hyperchromatic nuclei and a prominent chromatin ring, were arranged as sheets interspersed by dense collagen bundles. The cytoplasm contained distinct metachromatic granules that on ultrastructure were considered typical of avian mast cells. There were metastases to the lung (70).

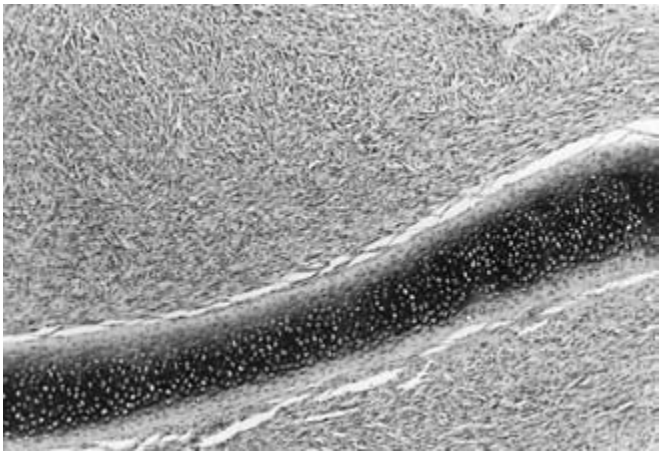
A basal cell carcinoma in the neck of a parrot consisted of lobules and nests of polygonal cells lacking intercelluar bridges. There was a tendency at the edge of the nests to form a palisade-like pattern reminiscent of feather barbs (146).

Atypical pox lesions of the feathered areas of broiler chickens may mimic dermal squamous cell tumors (44), and some skin lesions may be a combination of both dermal squamous cell carcinoma and cutaneous pox (45). Note that in the older literature atypical pox referred to poxvirus infection of the larynx and trachea. Nodular cutaneous wart-like, papular or deep craterous nodular lesions of feathered regions have been observed in association with poxvirus in several avian species, including pigeons, canaries (73, 117, 122) and flamingos (7). In these lesions pox inclusions are abundant within the cytoplasm of the superficial epithelial cells of hypertrophied and hyperplastic adnexal structures such as feather follicles that are penetrating deep into the dermis. These lesions tend to persist for a long period and are frequently biopsied for investigation of suspect neoplasia.

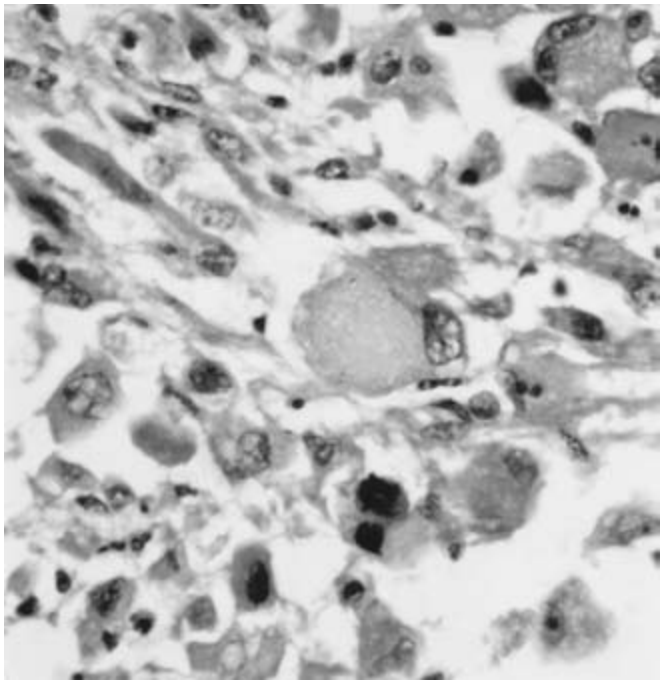
Musculoskeletal System

Leiomyoma and Leiomyosarcoma

Leiomyomas of the ventral ligament of the oviduct are common in laying hens (see "Reproductive System"), and equivalent tumors in other sites are histomorphogically similar. Leiomyomas were observed by the author in the intestinal wall of a commer-



15.99. Leiomyoma of the trachea of broiler chicken. Bundles and whorls of smooth muscle fibers in the lamina propria as shown in upper portion and penetrating between the cartilagenous rings and into the adjacent adventitia (lower right). H & E, ×100.



15.100. Rhabdomyosarcoma. Some cells are strap-like, whereas others are large and polyhedral. Their cytoplasm is eosinophilic. Some cells have multiple nuclei. H & E, ×360. (Courtesy of *Avian Pathology*)

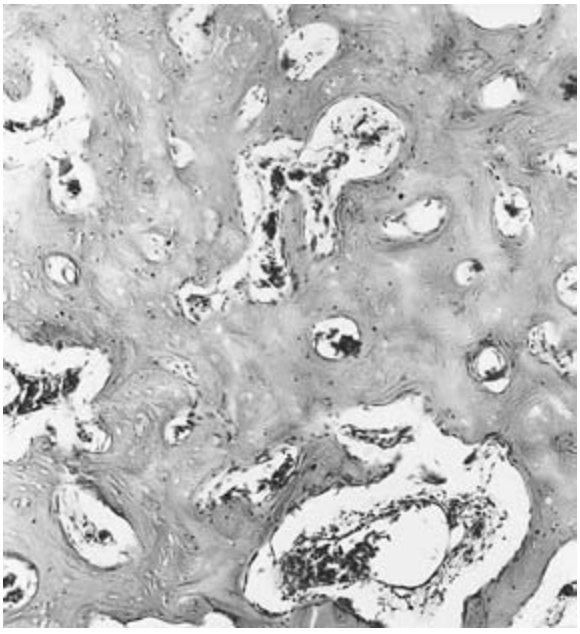
cial duck and a freckled duck (120), in the gizzard musculature of a chicken (121), in the tracheal ring of a broiler (122) (Fig. 15.99), and attached to the pancreas of pigeons (119). There are a few reports of leiomyosarcomas involving the intestinal wall (2), ovary (81), and tracheal muscle (26) of chickens. Leiomyomas and leiomyosarcomas are rare in other avian species (119, 127, 136).

Rhabdomyosarcoma

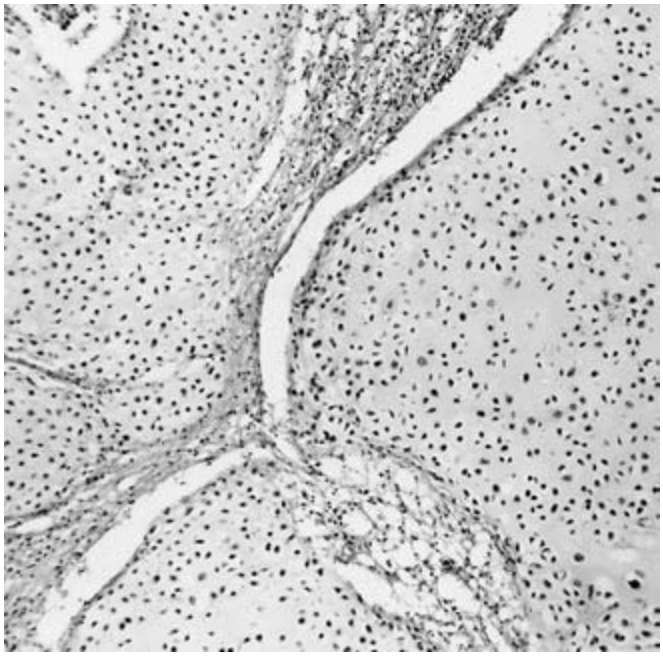
Rhabdomyosarcomas tend to be soft, poorly encapsulated and prone to necrosis and hemorrhage. The pectoral and sartorius-gracilus muscles were commonly involved, and also the heart (26). Metastases to the lung have been reported (88, 121). Histologically there are irregular bundles of interlacing cells, some of which exhibit a typical racquet or star shape, and multinucleated cells (Fig. 15.100). The cytoplasm is intensely eosinophilic, but cross striations are difficult to detect with either polarized light or phosphotungstic acid stain (25, 106). They also have been described in budgerigars (90, 119).

Osteoma and Osteosarcoma

Osteomas and osteosarcomas are uncommon tumors of poultry (25). Campbell and Appleby (26) described two osteomas, eight osteosarcomas, and one osteoclastoma in broilers, and similar tumors have been reported in other avian species (110, 119). Osteosarcomas may be composed of abundant mineralized trabecular bone, although in some cases there may be a much more cellular tumor composed of spindle-shaped cells and poorly mineralized trabeculae. Even in such cases, some foci of ossification usually can be found. Osteosarcomas may metastasize to the lungs. Multipotent mesenchymal tumors usually occur on the extremities of long bones and contain solid masses of dysplastic bone, islands of cartilage, dense drifts of fibrous tissue and foci of myxomatous tissue. These are frequently described as osteosarcomas. Osteomas are well-circumscribed and composed of disorganized bony trabeculae (Fig. 15.101). A tumor with both osteoid and cartilagenous elements involving the tracheal rings of a lovebird has been described (154).



15.101. Osteoma showing thick irregular trabeculae. H & E, ×90. (Courtesy of *Avian Pathology*)



15.102. Multifocal chondroma of the footpad of a goose showing lobules of cartilage separated by fibrovascular trabeculae. H & E, ×75. (Courtesy of *Avian Pathology*)

Chondroma and Chondrosarcoma

Chondromas of poultry are rare. Multifocal chondromas of the plantar aspect of the footpads of nine geese, ducks, and other anseriformes, and other mesenchymal tumors (3 myxomas and an osteoma) in the footpads of other Anseriformes were described by the author (119). The etiology of these was not determined, but approximately 10% of two flocks of wild mallards were affected. The chondromas were characterized by lobules of chondrocytes separated by trabeculae (Fig 15.102).

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Chapter 16

Salmonella Infections

Introduction

Richard K. Gast

Infections with bacteria of the genus *Salmonella* are responsible for a variety of acute and chronic diseases in poultry. These diseases continue to cause economically significant losses in many nations and absorb a large investment of resources in testing and control efforts in others. Infected poultry flocks are also among the most important reservoirs of salmonellae that can be transmitted through the food chain to humans. Poultry producers are faced with intensifying pressures from public health and regulatory authorities to protect consumers from illness transmitted by contaminated poultry meat and eggs. Poultry and poultry products are consistently among the leading animal sources of salmonellae that enter the human food supply. This circumstance is partly due to the high prevalence of *Salmonella* infections in poultry, but also reflects both the immense numbers of commercially raised chickens and turkeys and the application of extensive and active programs for identifying infected poultry flocks and products. The increasingly international scope of the modern poultry industry has created new and more complex opportunities for the spread of *Salmonella.*

The genus *Salmonella* (of the family Enterobacteriaceae), named for USDA veterinary bacteriologist Daniel E. Salmon (1850–1914), consists of more than 2500 serologically distinguishable variants (or serovars) that are frequently named for the place of initial isolation. Although recent taxonomic refinements have indicated that all salmonellae associated with poultry are members of a single genetically defined species—*Salmonella enterica*—the distinctions between the various serovars of *Salmonella* are often epidemiologically significant. Accordingly, *Salmonella* isolates are still most often described by reference to their traditional serotype nomenclature.

Infections of poultry with salmonellae can be grouped into three categories, each of which is the subject of a separate section of this chapter. The first section discusses infections with the two nonmotile serovars, *S.* Pullorum and *S.* Gallinarum*,* which are generally host-specific for avian species. Pullorum disease, caused by *S.* Pullorum*,* is an acute systemic disease of chicks or poults. Fowl typhoid*,* caused by *S.* Gallinarum*,* is an acute or chronic septicemic disease that most often affects mature birds. Both of these diseases have been responsible for serious economic losses to poultry producers in the past, and have been addressed by the implementation of extensive testing and eradication programs.

The second section of this chapter discusses infections with the numerous motile and non-host-adapted *Salmonella* serotypes referred to collectively as paratyphoid salmonellae. Found nearly ubiquitously in wild and domestic animals, this diverse group of serotypes is principally of concern as a cause of food-borne disease in humans. Although paratyphoid infections of poultry are very common, they seldom cause acute systemic disease except in highly susceptible young birds subjected to stressful conditions. More often, paratyphoid *Salmonella* infections of chickens and turkeys are characterized by asymptomatic and sometimes persistent colonization of the intestinal tract and internal organs, potentially leading to contamination of the finished carcass. Some serotypes, especially *S.* Enteritidis*,* can be deposited in the contents of clean and intact eggs laid by systemically infected hens. Because there are so many potential sources of introduction of paratyphoid salmonellae into poultry flocks, effective strategies for controlling these organisms require the combined and sustained implementation of a comprehensive set of risk reduction practices throughout the production continuum.

The third section of this chapter discusses infections with *S. enterica* subpsecies *arizonae,* a motile serotype which was formerly designated *Arizona hinshawii*. This organism, although biochemically distinct, causes an acute septicemic disease that is not clinically distinguishable from other *Salmonella* infections. Arizonosis has re-emerged as an economically significant disease of young turkeys in recent years.

Pullorum Disease and Fowl Typhoid

H. L. Shivaprasad and P. A. Barrow

Introduction

Pullorum disease and fowl typhoid in terms of history, clinical signs, epizootiology, lesions, and control and eradication procedures have many similarities. However, differences have been reported for these two diseases, and they are caused by different species of bacteria (i.e, *Salmonella* Pullorum and *S.* Gallinarum, respectively). Recently, these two bacteria have been placed in a single species. *S. enterica* subsp. *enterica* serovar Gallinarum-Pullorum, but debate continues as to whether they are single or different taxa within the same serovar.

Pullorum disease (PD) and fowl typhoid (FT) are septicemic diseases affecting primarily chickens and turkeys, but other birds such as quail, pheasants, ducks, peacocks, and guinea fowl are also susceptible. Both diseases can be transmitted through the egg by transovarian infection. *S. enterica* subsp. *enterica* Pullorum-Gallinarum is highly host adapted and seldom causes significant clinical signs, morbidity, or mortality in hosts other than chickens and turkeys.

Definition and Synonyms

Bacillary white diarrhea was a term used to designate PD before 1929, but the term *pullorum disease* has since gained universal acceptance.

Economic Significance

The elimination of PD and FT from commercial flocks in the United States in the mid-1900s was largely a result of the pullorum-typhoid control program, the National Poultry Improvement Plan (NPIP), instituted by a voluntary organization (10). Similar government-backed approaches contributed to reductions in Europe. Even though PD is rare in commercial chickens, the disease still occurs in backyard flocks (9, 56, 142, 167). The major economic cost from PD over the last 20 years has been the cost involved in testing breeding flocks of chickens and turkeys to ensure that they are free of the infection. Both diseases remain of considerable economic significance in those countries which have only recently begun to intensify their industries and where extensive breeding remains important or where the ambient conditions are such that environmental infection is difficult to control through improvements in housing.

Public Health Significance

Rare cases of PD in humans have resulted from massive exposure following the ingestion of contaminated foods or experimental challenge (110, 115). The clinical signs are characterized by a rapid onset of acute enteritis, followed by prompt recovery without treatment. *S.* Gallinarum is rarely isolated from humans and is of little public health significance (9, 124). According to a Centers for Disease Control and Prevention report (8), 18 *S.* Pullorum isolates and 8 *S.* Gallinarum isolates were reported out of a total of 458,081 *Salmonella* isolates from humans between 1982 and 1992. Experimental reproduction of salmonellosis with four strains of *S.* Pullorum in humans with large numbers (billions) of bacteria produced only transient clinical signs followed by prompt recovery (115).

History

Only the salient features of these two diseases are provided here. Further information on the history of these two diseases can be found in previous editions of this book, as well as in a historical review by Bullis (39).

The etiologic agent of PD was described by Rettger in 1899, and the disease was called fatal septicemia of young chicks (128). Later, the disease was designated as bacillary white diarrhea to distinguish it from other diseases of chicks (129). At that time PD was widespread in the United States and in many other countries throughout the world. Mortality associated with this disease in chicks ranged up to 100% (130), seriously threatening the chicken industry. Between 1900 and 1910, PD was shown to be an egg-borne infection. In 1913, the practical application of the macroscopic tube agglutination antibody test for detection of carriers of the organism was described (86). Standard methods of diagnosis of PD in barnyard fowl were formulated by the Conference of Research Workers in Animal Diseases of North America and later adopted by the USA Livestock Association, now the United States Animal Health Association (USAHA), in 1932 (5, 6). A modified whole blood test method in which stained antigen is used was developed in 1931 (137). It has been widely used because of its simplicity.

The NPIP, established in 1935, is administered by state agencies cooperating with the USDA, and was designed in part to control PD in chickens. Pullorum disease was first recognized in turkeys in 1928 (74), and by 1940, the disease was widespread in turkeys and responsible for severe economic losses. A National Turkey Improvement Plan, similar to NPIP, was organized in 1943. A series of modifications of these plans during a number of years has helped in the eradication of PD in commercial poultry.

Fowl typhoid, a disease very similar to PD, was first recognized in 1888, even before PD (91). Initially, the causative agent was named *Bacillus gallinarum,* later changed to *B. sanguinarium* and later to *S. gallinarum* (91). The name fowl typhoid was applied in 1902, and it was soon used in other parts of the world such as Germany and Holland. Control of FT was included in the NPIP in 1954. This resulted in the inclusion of FT in the same category as PD and is one of the main reasons for the eradication of FT in commercial poultry and its low incidence in all poultry.

The control and reduction of FT and PD have been suggested to be one of the reasons for the emergence of *S.* Enteritidis in the 1980s (126). Whether this is true or not remains to be seen.

Etiology

Classification

Pullorum disease and FT are caused by *S.* Pullorum and *S.* Gallinarum, respectively. However, these two bacteria have been placed in a single species, *S. enterica* subsp. *enterica* serovar Pullorum-Gallinarum of the family Enterobacteriaceae, which is highly host adapted. It is one of the few members of the genus that is non-motile and belongs to the serogroup D according to the Kauffman White scheme. Classification of the etiology of PD and FT has been confusing and has changed in the last few years from separate species of *S. pullorum* and *S. gallinarum* to a single species. *Bergey's Manual* once used the designation *Salmonella gallinarum* for the etiology of PD and FT, but more recently the designation of *S. gallinarum-pullorum* has been used. In some classification schemes, the etiology of PD and FT are given different serovar status (*S. enterica* subsp. *enterica* serovar Pullorum and *S. enterica* subsp. *enterica* serovar Gallinarum, respectively, as well as *S. enterica* serovar Gallinarum biovars gallinarum and pullorum) because of biochemical differences and epizootiology. For simplicity in this chapter, the etiology of PD will be termed *S.* Pullorum, and the etiology of FT will be termed *S.* Gallinarum.

Nonmotile salmonellae such as *S.* Pullorum and *S.* Gallinarum are monophyletic, and the most recent common ancestor is nonmotile (102). Since diverging from this ancestor, the Pullorum lineage has evolved more rapidly than the Gallinarum lineage as shown by multilocus enzyme electrophoresis and estimation of the chromosomal genotypic diversity (102). Using multilocus enzyme electrophoresis, it was shown that *S.* Enteritidis, a polyphyletic serotype, is closely related to *S.* Pullorum and *S.* Gallinarum (155). Genetic differences were also found between isolates of *S.* Pullorum isolated prior to the 1980s and isolates of *S.* Pullorum isolated during the 1990s based on random amplified polymorphic DNA assay (52). This has been supported more recently by microarray comparisons of whole genomic DNA (43, 125), and full analysis of the recently sequenced genomes (www.sanger.ac.uk/Projects/Salmonella) will enable extensions of these comparisons. Chromosomal rearrangements have also been observed in these serovars which, it has been speculated, are related to host adaptation (103, 184). Some differences also exist between the two taxa including deletions in Pullorum strains in the Tor respiration system and in the std fimbriae and chemotaxis genes in Gallinarum strains (125) and more unusual strains with large deletions were also seen.

Gallinarum strains can also be differentiated from Pullorum strains by PCR based assays reflecting local genomic sequence differences such as those in the rfbS (140) and glgC genes (111).

Morphology and Staining

The organisms are Gram-negative, non-sporogenic, and facultatively anaerobic. They are slender rods measuring 0.3 to 1.5 mm in width and 1.0 to 2.5 mm in length. The bacilli mostly occur singly, but occasionally two or more can be found to be united. Both *S.* Pullorum and *S.* Gallinarum are considered as nonmotile. However, motility and flagellation have been shown in *S.* Pullorum grown on special types of solid media (45, 68, 77). However, other workers were unable to induce motility in *S.* Pullorum when grown on Hektoen agar (44).

Growth Requirements

S. Pullorum and *S.* Gallinarum grow readily on beef agar, broth, or other nutrient media. They are aerobic or facultatively anaerobic and grow best at 37°C. The organisms will grow in selective enrichment media such as selenite-F and tetrathionate broths, and differential plating media such as MacConkey, bismuth sulfite, and brilliant green agars. It has been shown that *S.* Pullorum occasionally fails to grow on certain selective media such as brilliant green or salmonella-shigella agar but grows satisfactorily on bismuth sulfite and MacConkey agars (41). *S.* Pullorum appears to grow slower than *S.* Gallinarum, and this has been attributed to its inability to oxidatively assimilate a variety of amino acids (156).

Colony Morphology

Colonies of *S.* Pullorum and *S.* Gallinarum on meat extract or infusion agar (pH 7.0–7.2) appear as small, discrete, smooth, blue-gray or grayish white, glistening, homogenous, and entire. The growth of *S.* Pullorum and *S.* Gallinarum is luxuriant and markedly translucent on liver infusion agar. Covered colonies remain small (1 mm or less), but isolated colonies may have a diameter of 3–4 mm or more. Surface markings may appear as the colonies increase in size and age, but as a rule, young colonies in a heavily seeded plate change little with age. Occasionally, morphologically abnormal strains are encountered. Inoculation of gelatin slants yields grayish-white surface growth with filiform growth in the stab and no liquefaction. Growth in broth is turbid with heavy flocculent sediment.

Biochemical Properties

More similarities than differences are identified between *S.* Pullorum and *S.* Gallinarum in their biochemical properties (33, 50, 163). Both organisms can ferment arabinose, dextrose, galactose, mannitol, mannose, rhamnose, and xylose to produce acid, with or without gas production. Substances not fermented include lactose, sucrose, and salicin. One important biochemical difference between these two organisms is that *S.* Gallinarum ferments dulcitol, whereas *S.* Pullorum does not. Also, *S.* Pullorum only occasionally ferments maltose. The major difference between the two organisms is, however, that *S.* Pullorum cultures produce rapid decarboxylation of ornithine, whereas cultures of *S.* Gallinarum do not. In addition, *S.* Gallinarum uses citrate, D(2) sorbitol, L(2) fucose, D(2) tartrate, and cysteine hydrochloride gelatin (163). Some of these differences are helpful in differentiating the two organisms; however, variation in the characteristics of some strains can be observed occasionally, especially in regard to gas production.

Ribotyping by the use of the enzyme *Eco*RI has been suggested as an important tool to differentiate between *S.* Pullorum and *S.* Gallinarum (49). Also, profiles of whole-cell fatty acid methyl ester have been helpful for separation and identification of *S.* Pullorum and *S.* Gallinarum (133). More recently it has

been demonstrated that *S.* Pullorum can be differentiated from *S.* Gallinarum by the analysis of the phase 1 *flagellin* C gene (*flic*) by single-strand conformational polymorphism (98).

Susceptibility to Chemical and Physical Agents

In general, the susceptibility of *S.* Pullorum and *S.* Gallinarum is about the same as that of members of the paratyphoid groups (123, 153). They may survive for several years in a favorable environment, but they are less resistant than paratyphoid salmonellae to heat, chemicals, and adverse environmental factors. For example, *S.* Gallinarum was killed within 10 minutes at 60°C; within a few minutes by direct exposure to sunlight; in 3 minutes by 1:1000 phenol, 1:20,000 dichloride of mercury, or 1% potassium permanganate; and in 1 minute by 2% formalin (153). *S.* Gallinarum grown on agar cultures may rapidly lose their virulence. Orr and Moore (117) found that *S.* Gallinarum retained viability up to 43 days when subjected to daily freezing and thawing. Organisms in liver survived more than 148 days at –20°C, even though they were accidentally thawed twice. *S. Gallinarum* can survive in feces from infected chickens up to 10.9 days when kept in a range house and 2 days less in the open (148).

Antigenic Structure

Both *S.* Pullorum and *S.* Gallinarum possess the O antigens 1, 9, and 12. Variation involving antigen 12 occurs in *S.* Pullorum strains based on serologic testing, but there is no evidence for such strain variation in *S.* Gallinarum. However, DNA fingerprint analysis has raised doubts regarding such variation in the major somatic antigen 12 of *S.* Pullorum (180).

The first serologic evidence of antigenic variation in *S.* Pullorum strains was discovered when progeny infected with a new strain were negative for antibodies using a standard agglutination test. Sera from infected chicks agglutinated homologous-strain antigen but not standard-strain antigens. This antigenic variation, characteristic of *S.* Pullorum, was studied extensively (54, 55, 178, 185). The antigenic composition of *S.* Pullorum was shown to contain O antigens 9, 12_1, 12_2, and 12_3, but the quantity of antigens 12_2 and 12_3 varies with strains. Standard strains contain a large amount of 12_3 and a very small amount of 12_2, but in variant strains, the content of the two antigens is reversed. Extensive examination of individual colonies, sometimes through successive transfers, has been necessary to determine accurately the antigenic form of a culture. Most isolates tend to stabilize during passage on artificial media. Standard-form cultures usually contain a small percentage of 12_2-predominant colonies even after long artificial cultivation. Variant forms of cultures are often pure or nearly pure for 12_2 and 12_3 factors. Colonies of intermediate strains are usually mixtures of 12_2- and 12_3-predominant colonies or rarely are uniform and contain appreciable amounts of both factors in individual colonies. Strains may also vary in content of the O–1 antigen.

Early reports indicated that as many as one-third of the *S.* Pullorum isolates from some areas of the United States were of the variant type; by 1950, only 13% of total isolates were of that type (177), a reduction believed to be the result of the extensive use of polyvalent testing antigens.

Tests to differentiate standard, intermediate, and variant types of *S.* Pullorum have been described (174, 175). Phage typing of *S.* Pullorum can be used for type identification, epidemiologic investigations, and genetic studies (50, 164, 165).

Virulence Factors

S. Pullorum and *S.* Gallinarum, being Gram-negative bacteria, have endotoxin. Unfortunately, this has not been studied extensively. *S.* Pullorum contains a thermostable toxin to which rodents, but not chicks, are susceptible. Similarly, *S.* Gallinarum contains a toxin that was lethal to rabbits (152). Endotoxins from *S.* Gallinarum can cause clinical signs within a few hours after intravenous injection in chicks (151). Most of the clinical signs subsided within 24–48 hours. Storing *S.* Gallinarum at 275 or 220°C did not have any effect on the subsequent pathogenicity test results (151).

Like most pathogenic microorganisms, *S.* Gallinarum, and probably *S.* Pullorum, may lose virulence rapidly during propagation and passage on artificial media; hence, cultures should be passaged serially in their natural host, the chicken, before testing the pathogenicity of the organisms. Pathogenicity of such cultures is best maintained in the lyophilized or frozen state. Passage on artifical media by different investigators have found wide variation in virulence among cultures of *S.* Gallinarum.

An 85-kb plasmid plays a role in the virulence of *S.* Pullorum and *S.* Gallinarum in chickens (18, 21, 50, 112). Plasmids of sizes other than 85-kb, either alone or in combination, or isolates absent in plasmids have also been reported in *S.* Gallinarum (4, 112).

As might be expected from *Salmonella* species causing systemic typhoid-like diseases, *Salmonella* Pathogenicity Island 2 is essential to virulence and survival and multiplication intracellularly (87, 173, 139) although the contribution of SPI1 encoding genes required for invasion of nonprofessional phagocytes is unclear. Other virulence gene clusters can also be identified although have not yet been fully characterized.

Certain genes are responsible for efficient adherence and entry of various salmonellae into cultured epithelial cells (2). When a mutated version of one of these genes, *invH,* was introduced into different *Salmonella* strains, some of these salmonellae (including *S.* Gallinarum) were rendered deficient for adherence and invasion of cultured cells. In one study, significant variability was observed among *S.* Pullorum isolates in their ability to invade avian epithelial cells despite the presence of *Salmonella* invasion genes *spvB* and *invA* (52). However, the relative role of the various fimbriae expressed by these and other *Salmonella* species has not been fully assessed. Genes homologous to K88 genes *fae*H and *fae*I have been demonstrated on the virulence plasmid of *S.* Gallinarum, which may influence intestinal invasion (132) and which may be equivalent to the Pef fimbriae of *S.* Typhimurium. Sef fimbriae are also produced (127).

Toxins produced by *Salmonella* serovars continue to be reported. Amongst these are a hemolysin (1) considered to be a regulatory gene rather than a structural toxin.

Pathobiology and Epidemiology

Incidence and Distribution

Pullorum disease and FT are worldwide in distribution (15, 32, 36, 47, 78, 79, 84, 98, 104, 106, 109, 112, 116, 136, 145, 146, 185, 186). Pullorum disease is rare in commercial poultry in the United States and perhaps in other parts of the world such as Canada, Australia, Japan, and Western Europe. Pullorum disease is also less commonly reported from backyard chickens in the United States (9). Except for one epizootic of PD in commercial chickens in the United States during 1990–1991, there have been no recent outbreaks (85, 134). This epizootic involved a single integrated broiler operation in five states (Delaware, Maryland, North Carolina, Alabama, and Florida). The outbreak involved 18 breeder flocks and more than 261 broiler rearing facilities.

No outbreaks of FT in commercial poultry in the United States have been reported since 1980 (7, 121). Canada and several European countries have a low incidence or absence of FT. Mexico, Central and South America, Africa, and the Indian subcontinent continue to report PD and FT in poultry flocks (16, 32, 36, 84, 104, 106, 109, 112, 136, 145, 146). Recently, a few outbreaks of FT have been reported in commercial poultry in Denmark and Germany as a result of importation from Eastern Europe (51).

Natural and Experimental Hosts

Chickens are the natural hosts for both *S.* Pullorum and *S.* Gallinarum; however, naturally occurring outbreaks of PD and FT have been described in turkeys, guinea fowl, quail, pheasants, sparrows, and parrots (120, 123, 143, 153). In addition, naturally occurring outbreaks of PD have been described in canaries and bullfinches, and FT has been described in ring doves, ostriches, and peafowl. The susceptibility of ducks, geese, and pigeons to *S.* Gallinarum has been variable, but most breeds used currently appear to be resistant to this pathogen. Ducks were found to be resistant to experimental infection with *S.* Pullorum and *S.* Gallinarum (19, 38).

Significant differences in susceptibility to PD among breeds of chickens have been described (40, 138). The lighter breeds, particularly leghorns, appear to be more resistant than the heavy breeds. PD-resistant and PD-susceptible lines of Rhode Island reds, New Hampshires, and crosses between the two were developed based on the selection for high and low body temperature during the first 6 days of life (81). Enormous differences in resistance to *S.* Pullorum and *S.* Gallinarum have also been shown in inbred lines of white leghorn chickens (35) much of which can be attributed to one locus, designated *SAL1* although TLR4 and *NRAMP1* also contribute (80, 101, 107). It appears that a greater percentage of females than males stay as reactors, probably due to local infection being sequestered in the ovarian follicles.

Pullorum disease has been described as a naturally occurring or experimental infection in mammals including chimpanzees, rabbits, guinea pigs, chinchillas, pigs, kittens, foxes, dogs, swine, mink, cows, and wild rats. *S.* Gallinarum was able to be cultured for up to 121 days from the feces of experimentally infected rats (13). Human salmonellosis caused by *S.* Pullorum occasionally has been reported (8, 110, 115). The basis of host adaptation is unknown (42) although it is apparent that it is predominantly expressed at the level of the macrophage-monocyte series (17).

Age of Hosts Commonly Infected

Mortality from PD usually is confined to the first 2–3 weeks of age. Acute infections in older chickens, particularly among brown egg-producing strains, have been reported occasionally. Similarly, mortality due to PD in semi-mature and mature turkeys has been observed. A certain percentage of chickens and turkeys that survive the initial infection become carriers with or without the presence of lesions.

Although FT is frequently referred to as a disease of adult birds, there have been reports of high mortality in young chicks (22, 25, 97, 108, 183). Fowl typhoid can cause mortality as high as 26% in chicks during the first month of life. Mortalities of 65% and 100% within 11 days after inoculation of one-day-old broiler chicks with 10^4 CFU and 10^8 CFU per ml of *S.* Gallinarum, respectively, have been reported (183). As in PD, FT losses begin at hatching time; however, in FT they also continue to laying age. Certain strains of *S.* Gallinarum produce lesions in chicks indistinguishable from those associated with PD (154). Therefore, as a result of experimental and field observations we can conclude that chicks and poults are highly susceptible to *S.* Pullorum and *S.* Gallinarum, whereas adults are more susceptible to *S.* Gallinarum.

Transmission

Like many other bacterial diseases, PD and FT can be transmitted in several ways. The infected bird (reactor and carrier) is by far the most important means of perpetuation and spread of the organism. Birds may infect not only their own generation by horizontal transmission, but also succeeding ones through egg transmission. Egg transmission may result from contamination of the ovum following ovulation (22, 25), but localization of *S.* Pullorum or *S.* Gallinarum in the ovules before ovulation is likely and probably constitutes the chief mode of vertical transmission. The relative contribution of vertical transmission in the two organisms is unclear since it is easy to establish persistent infections and egg transmission with *S.* Pullorum (31) but much less easy with *S.* Gallinarum and it may be that horizontal transmission is more important in this highly virulent organism where experimental infection generally either results in clinical disease and mortality or no infection depending on the genetic background of the host. Egg transmission has been influenced by levels of antibodies in the yolk (169). Maternal antibodies against *S.* Pullorum may be critical in the prevention of embryonic mortality in infected eggs, thus allowing successful egg transmission.

The exact mechanism surrounding persistent infection and carriage which results in infection of the reproductive tract is still unclear. However, it is known that *S.* Pullorum persists within macrophages in the spleen during the carrier state (170) and although numbers gradually reduce they are not eliminated (31) and at sexual maturity the numbers increase as a result of reduced capacity of T cells to respond specifically and nonspecifically to

antigens (171), probably following increases in sex hormones. The reduced immune responsiveness enables the bacteria to spread to the reproductive tract.

Transmission through shell penetration and feed contamination by *S.* Pullorum has been reported but appears to be of minor importance (176). The number of eggs infected with *S.* Pullorum or *S.* Gallinarum can be as high as 33% of the total laid by an infected hen. Contact transmission of infected chicks or pullets can be an important route of dissemination of *S.* Pullorum and *S.* Gallinarum. This can happen in the hatcher and can be partially prevented only by formaldehyde fumigation (75). Mortality as high as 60.9% of the exposed flock due to *S.* Gallinarum has been reported (66). Transmission may also occur within a flock as a result of cannibalism of infected birds, eating of infected eggs, and through wounds on the skin. Feces from infected birds are also a source of bacteria. Contaminated feed, water, and litter can also be sources of both *S.* Pullorum and *S.* Gallinarum. Attendants, feed dealers, chicken buyers, and visitors who move from house to house and from farm to farm may also spread these diseases unless precautions are taken to disinfect footwear, hands, and clothing. Similarly, trucks, crates, and feed sacks may also be contaminated. Wild birds, mammals, and flies may be important mechanical spreaders of the organisms.

Clinical Signs

PD and FT are primarily diseases of chicks and poults. However, FT is a more significant disease in growing and adult chickens and turkeys than PD. The signs noted in young chicks and poults associated with both diseases are very similar as a result of the transovarian transmission of these diseases. Occasional cases of PD can be subclinical, even though the disease may originate by egg transmission.

Chicks and Poults

If birds are hatched from *S.* Pullorum or *S.* Gallinarum infected eggs, moribund and dead birds may be observed in the incubator or within a short time after hatching. The birds can manifest somnolescence, weakness, depressed appetite, poor growth, and adherence of chalky white material to the vent. Death may soon follow. In some cases, evidence of PD is not observed until 5–10 days after hatching, but the disease gains momentum during the following 7–10 days. Mortality usually peaks during the second or third week of life. In these situations, the birds exhibit lassitude and an inclination to huddle together under heaters, having droopy wings, and distorted body appearance.

Labored breathing or gasping may be observed as a result of extensive involvement of the lungs due to PD. Survivors may be greatly retarded in their growth and appear underdeveloped and poorly feathered. These birds may not mature into vigorous or well-developed laying or breeding birds. Flocks that have passed through a serious outbreak usually have a high percentage of carriers at maturity.

Blindness, as well as swelling of the tibiotarsal and the humeroradial and ulnar articulations, due to *S.* Pullorum infection in chicks has been described (26, 57, 59, 85, 109, 134). In certain instances, a relatively high incidence of infection in the joints, which can produce lameness and obvious joint enlargement, can occur in chicks. In the 1990–1991 outbreaks of PD in the eastern part of the United States, swelling of the hock joint, due to synovitis, was commonly seen (134). Similar lesions in turkey poults have also been reported. This may suggest that some strains of *S.* Pullorum may have a tropism for these sites.

Growing and Mature Fowl

Infected birds may not exhibit any signs and cannot be detected by their physical appearance, especially in the case of PD. Acute outbreaks of FT in chickens may begin by a sudden drop in feed consumption, with birds being droopy, showing ruffled feathers and pale and shrunken combs. Other signs, such as a drop in egg production, decreased fertility, and diminished hatchability, can sometimes be observed in both PD and FT, depending upon the severity of infection. Death may occur within 4 days of exposure, but usually occurs after 5–10 days. There may be an increase in body temperature of 1–3 degrees within 2–3 days after exposure. In some cases of PD in semi-mature and mature flocks, the predominant clinical signs include anorexia, diarrhea, depression, and dehydration (56).

Clinical signs due to PD and FT in turkeys may consist of thirst, inappetence, listlessness, a tendency to separate from healthy birds, and green to greenish-yellow diarrhea. However, deaths may occur with no prior clinical signs, but body temperature may increase several degrees initially. The first outbreak on a farm usually causes the highest mortality followed by intermittent recurrence and less severe losses (76).

Morbidity and Mortality

Both morbidity and mortality are highly variable in chickens and are influenced by age, strain of bird, nutrition, flock management, concurrent diseases and route and dose of exposure. Mortality from PD may vary from 0 to 100%. The greatest losses usually occur during the second week after hatching, with a rapid decline between the third and fourth week of age in PD. Mortality ranging from 10 to 93% due to FT have been reported in chicks (72). One hundred percent mortality in 11 days was reported in broiler chicks after they were inoculated at one day of age with 10^8 CFU per ml of *S.* Gallinarum (183).

Morbidity is often much higher than mortality with some of the affected birds recovering spontaneously. Birds hatched from an infected flock and raised on the same premises exhibit less mortality than those subjected to the stress of shipping. Losses may be as severe in turkeys as in chickens.

Pathology

Gross Lesions

Descriptions of gross and microscopic lesions associated with PD and FT are sporadic. Some of the earliest descriptions were those by Rettger (129, 130). Since then, there have been many reports in various species of birds, primarily involving chickens and turkeys, but also in pheasants, quail, and guinea fowl (22, 27, 46, 53, 56, 57, 65, 72, 73, 74, 75, 95, 96, 108, 109, 120, 141, 142, 143, 160, 183, 185).

Chicks

In peracute cases of PD and FT, birds that die suddenly in the early stages of brooding may show no gross lesions. In acute cases, enlarged and congested liver, spleen, and kidneys may be seen (Fig. 16.1A). Livers may have white foci of 2–4 mm in diameter (Fig. 16.1B). The yolk sac and its contents may or may not reveal any abnormalities, but in protracted cases, interference with yolk absorption may occur. In such cases, the yolk sac contents may be of creamy or caseous consistency. Occasionally, those birds with respiratory signs may have white nodules in the lung (Fig. 16.1H), and white nodules, resembling Marek's disease tumors, may be present in the cardiac muscle (Fig. 16.1C) or pancreas. Occasionally, nodules in the heart may become so large they distort the shape of the heart (Fig.16.1D). This, in turn, may lead to chronic passive congestion of the liver and ascites. The pericardium may be thickened, and the pericardial sac may contain yellow serous or fibrinous exudate. Similar nodules may be present in the muscle of the ventriculus (gizzard) (Fig. 16.1E) and occasionally in the wall of the ceca and rectum. The ceca may contain caseous cores. Some birds may exhibit swollen joints containing yellow viscous fluid (27) (Fig. 16.1G); this was one of the most commonly reported gross lesions in the 1990–1991 outbreak of PD in commercial broilers in the United States (134). Among the joints, the hock joint is most commonly involved, but other joints such as the wing joint and the footpad may be affected. Other changes that can be seen include exudate in the peritoneal cavity, thickening of the wall of the intestine, and exudate in the anterior chamber of the eye.

Splenomegaly, gray necrotic foci with petechial hemorrhages in lungs, and pale or discolored livers were observed in bobwhite quail inoculated with *S.* Pullorum (38). In young pheasants, yolk sac infection, pneumonia, hepatitis, and typhlocolitis were the most common lesions associated with PD (120).

Adult Chickens

Lesions due to PD may be minimal in some birds, even though they may be active serologic reactors. Sometimes, only a minimal lesion, such as a small nodular or regressing ovarian follicle, can be found. However, the lesions found most frequently in chronic carrier hens with PD and FT are a few misshapen, discolored cystic or nodular ova among a few normal-appearing ovules (Fig. 16.1H and I). The involved ova may contain oily and caseous material enclosed in a thickened capsule. These degenerative ovarian follicles may be closely attached to the ovary, but frequently they are pedunculated and may become detached from the ovarian mass. In such cases, they may become embedded in the inner lining of the peritoneal cavity. Often, the oviduct contains caseous exudate in the lumen. Ovary and oviduct dysfunction may lead to peritoneal ovulation or oviduct impaction, which in turn may bring about extensive peritonitis and adhesions of the abdominal viscera. Fibrinous peritonitis and perihepatitis, with or without involvement of the reproductive tract, may sometimes be seen. Ascites also may develop, especially in turkeys. However, it may be difficult to culture *Salmonella* from advanced chronic lesions.

Frequently, pericarditis is observed. Changes in the pericardium, epicardium, and pericardial fluid depend on the duration of the disease. In some cases, the pericardium exhibits only a slight translucency, and the pericardial fluid may be increased in volume and turbid. In the more advanced stages, the pericardial sac is thickened and opaque, and the pericardial fluid is greatly increased in amount, containing considerable exudate. More advanced cases have chronic thickening of the pericardium and epicardium and partial obliteration of the pericardial cavity by adhesions. Occasionally, small cysts containing amber, caseous material may be found embedded in the abdominal fat or attached to the ventriculus and intestine. Frequently, the pancreas may have white foci or nodules.

In the male, testes may have white foci or nodules (65). Occasionally, caseous granulomas can be found in the lungs and air sacs (56).

Turkeys

Lesions due to PD and FT in turkeys are similar to those observed in chickens (74). Enlarged mahogany or brown-streaked livers, splenomegaly, areas of necrosis in the heart, and grayish lungs are characteristic lesions of FT in turkey poults. Ulceration throughout the small intestine and ceca is a common type of lesion in turkeys but uncommon in chickens. In adult carriers, there is a predilection of infection for the reproductive organs similar to that seen in chickens.

Ducks and Guinea Fowl

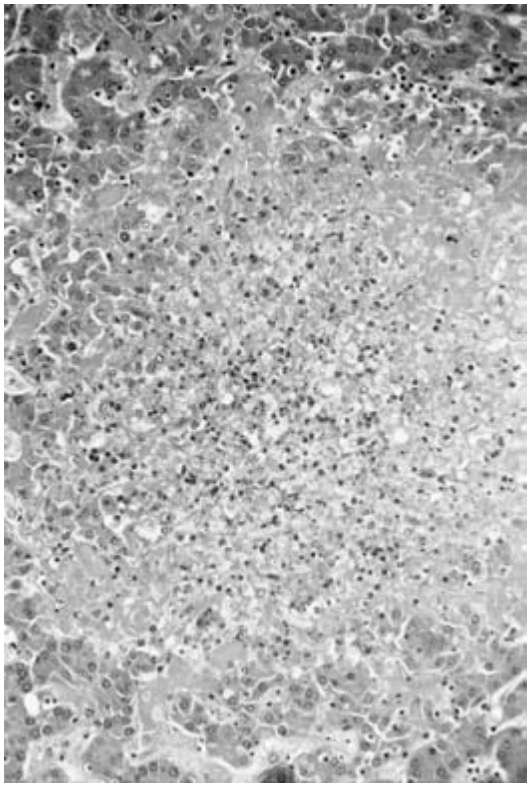
Lesions due to FT in ducklings and adult ducks are similar to those in chickens. In guinea fowl, FT lesions involve the respiratory tract and are characterized by congested lungs and increased mucus in the nasal cleft and trachea.

Histopathology

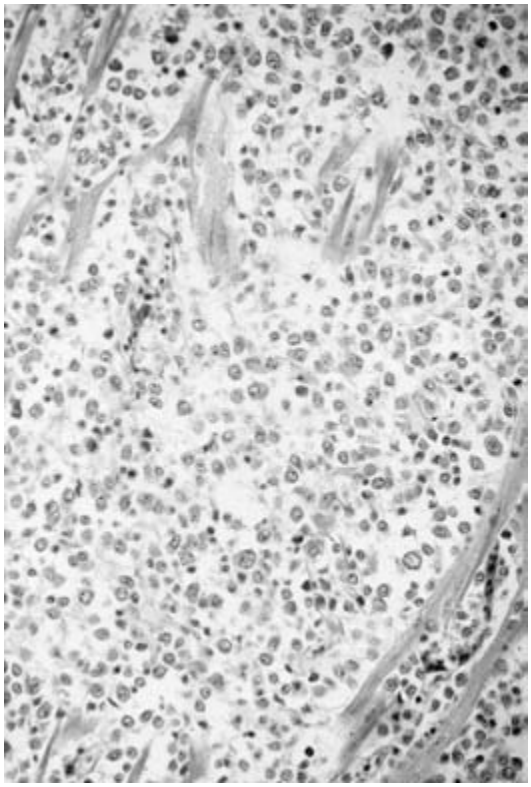
A very limited amount of information on microscopic lesions is available for PD or FT. Most of the PD lesions described are from field cases, which might have been complicated by other bacterial and/or viral agents (53, 160). There is one report on experimental pathology of FT in chicks (183).

In peracute cases of PD and FT, only severe vascular congestion in various organs, especially liver, spleen, and kidney, can be identified. In acute to subacute cases, there is multifocal necrosis of hepatocytes (Fig. 16.2) with accumulation of fibrin and infiltration of heterophils in the hepatic parenchyma. Periportal infiltration of heterophils mixed with a few lymphocytes and plasma cells can also be seen in the liver. In chronic cases, especially in cases in which there are large nodules in the heart, the liver will have chronic passive congestion with interstitial fibrosis. The spleen may have severe congestion or fibrin exudation of vascular sinuses in acute stages, and severe hyperplasia of the mononuclear phagocytic system cells in later stages. The ceca in young chicks may have extensive necrosis of the mucosa and submucosa, with an accumulation of necrotic debris mixed with fibrin and heterophils in the lumen.

However, the most characteristic microscopic lesions are in the heart and ventriculus. In the heart, they begin as necrosis of myofibers with infiltration of heterophils mixed with lymphocytes



16.2. Liver revealing focal degeneration and necrosis, ×51.



16.3. Myocardium from a *S.* Pullorum-infected chick showing infiltration with histiocytic-type cells, ×62.

and plasma cells. In later stages, these cells are replaced by massive numbers of uniform histiocytes (Fig. 16.3). These cells are fairly large, with irregular vesicular nuclei and faintly staining, foamy eosinophilic cytoplasm. They may be arranged in solid sheets, forming nodules that often protrude from the epicardial surface. These nodules, both grossly and histologically, can be confused with lymphoid tumors caused by Marek's disease virus and possibly retroviruses. A similar process can be seen in the ventriculus and pancreas. The lesions in the pancreas can be so severe that the normal architecture is obliterated.

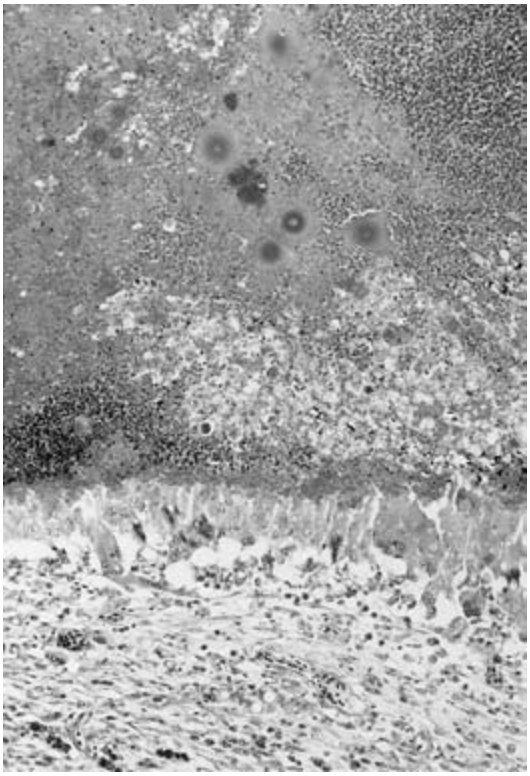
Other changes such as serositis of various organs including the pericardium, pleuroperitoneum, synovium, and serosa of the intestinal tract and mesentery can be seen in a high percentage of cases (160). In acute stages, these lesions can be associated with heterophils and fibrin, but in later stages, only lymphocytes, plasma cells, and histiocytes will be found.

Microscopic lesions in the ovary range from acute fibrinosuppurative inflammation to severe pyogranulomatous inflammation of the ovules (Fig. 16.4). The pyogranulomatous inflammation is characterized by infiltration of heterophils mixed with fibrin and bacterial colonies in the coagulated yolk material. In turn, the core is surrounded by successive layers of multinucleated giant cells and a mixed population of inflammatory cells that can include macrophages, plasma cells, heterophils, and lymphocytes. In males, degeneration, necrosis, and inflammation of the epithelial cells lining the seminiferous tubules can be seen (96). Other, but less common, changes are catarrhal bronchitis, catarrhal enteritis, and interstitial inflammation of the lungs and kidneys. Minimal and nonspecific changes in endocrine glands, such as the hypoplasia of thyroid and hypertrophy of adrenal and pituitary glands due to FT, have been described (61).

Pathogenesis of Infectious Process

Differences in the ability of various salmonellae, including *S.* Pullorum and *S.* Gallinarum, to survive and multiply in visceral organs (especially in spleen and liver) have been attributed to an unknown mechanism involving the mononuclear phagocyte system (MPS) of the host (17). Ducks may be resistant to *S.* Gallinarum due to the inherent inability of the bacteria to multiply in the MPS of ducks (19). Because of the ability of these bacteria to infect and multiply within the cells of the MPS of chickens and turkeys, it is probable that cell-mediated immunity to *S.* Pullorum and *S.* Gallinarum may play a role in recovery and resistance to infection in chickens and turkeys (171).

In one study, it was found that an *S.* Gallinarum infection of primary chicken cells caused a reduction in IL-1beta and no change in IL-6 (89). Modest increases in IL-ß were also found *in vivo* following vaccination with the 9R vaccine (172). The reduction in IL-1beta was interpreted as a lack of an inflammatory response resulting in systemic infection of *S.* Gallinarum (89). This, it was argued, was a strategic approach to facilitate invasion without stimulating the strong inflammatory responses associated with infection with most *Salmonella* strains (181, 182). Since signaling via TLR5 with flagella may be important, the absence of flagella



16.4. Ovule from an adult hen with pullorum disease showing fibrinosuppurative inflammation and bacterial colonies, ×20.

in these species may be highly significant (82). It is likely that these early responses are largely mediated through heterophilic granulocytes (92) which are not induced by these serovars unlike *S.* Enteritidis. In another study it was demonstrated that *S.* Pullorum preferentially targeted the bursa of Fabricius prior to eliciting inflammation in the intestine (73). In a study with *S.* Gallinarum in chickens, leucocytosis was observed on the third day PI but was followed by leukopenia that lasted for 10 days (93). This was followed by a leucocytosis as late as 21 days PI, and it was associated with heterophilia but a relative lymphopenia (93). Infection with *S.* Gallinarum may cause anemia (48) and an increase in sialic acid content in serum (94).

In most cases of FT, the organism is disseminated via intestinal lesions although the nature of this process and the cells involved are not understood.

Immunity

Very little information regarding immunity to PD and FT is available, due in part to the great success in the eradication of these diseases in commercial poultry. Chicks orally infected at 4 days of age did not produce detectable agglutinating antibodies until 20–40 days of age, but mature birds produced agglutinating antibodies within 3–10 days following infection. In chicks, maximum antibody production was not reached until 100 days after infection. The possible role of agglutinating antibodies in affecting the course of infection in the host is poorly understood.

It seems likely that with systemic diseases cell-mediated immunity is likely to be of major important in clearance (58). Little information is available specifically on *S.* Pullorum and *S.* Gallinarum. With S. Typhimurium, clearance is correlated with IFN-γ levels (24) and a strong T cell response (23). A recent study has also shown high IFN-γ levels following vaccination with the live 9R vaccine and strong lymphoproliferative responses (172). Although it is assumed that a strong Th1-type response is responsible for immunity the absence of studies involving Th2-type responses restricts analysis. It is thought that *S.* Pullorum induces a stronger Th2-type response which is responsible for the persistent infection and it is known that SPI2 secreted proteins are involved (173).

Diagnosis

A definitive diagnosis of PD or FT requires the isolation and identification of *S.* Pullorum or *S.* Gallinarum, respectively. A tentative diagnosis, however, can be made based on flock history, clinical signs, mortality, and lesions. Positive serologic findings can also be of major value in detecting infection; however, negative results should not be considered adequate for a definite diagnosis because of the delay of 3–10 days in the appearance of agglutinating antibodies following infection. Similarly positive results should be interpreted with caution because of cross-reactions with other serogroup D salmonellae such as *S.* Enteritidis (63, 64, 144, 168).

Isolation and Identification of Causative Agent

Acute PD and FT are characteristically systemic infections; causative organisms can be isolated from most internal organs. The liver, spleen, and ceca usually are involved and are the preferred organs to culture. Lesions may occur in lungs, heart, ventriculus, pancreas, or yolk sac, and these are also dependable samples for culturing. In mature birds, if lesions are present in the reproductive organs, ovarian follicles and testes can be cultured. Other sites for culturing are the peritoneum, synovial fluid, and the interior of the eye. Beef extract or infusion, or tryptose agar, in tubes or petri dishes, are all satisfactory for primary isolation. Enrichment broths or selective media may also be used if tissues are decomposed.

Birds with chronic PD or FT that are detected by serologic tests may or may not have gross lesions. If such birds are submitted to the laboratory as carriers, thorough culturing of internal organs is necessary. A detailed outline for testing such specimens is provided in the procedures manual of the NPIP (10). This procedure may be summarized as follows.

Grossly normal or diseased internal organs should be cultured directly on veal infusion (VI) and brilliant green (BG) agar plates and incubated for 48 hours at 37°C. In addition, portions of the internal organs should be pooled, ground, or blended in 10 times their volume of VI broth; 10 ml aliquots of the suspension are transferable to 100 mL of both VI and tetrathionate BG (TBG) broth and incubated for 24 hours at 37°C. The broths then are plated on VI and BG agar and incubated and examined after 24 and 48 hours. If contamination with proteus or pseudomonas is a problem, platings can be done on BG sulfapyridine (BGS) agar.

The digestive tract should be cultured using individual cotton swabs for the upper, middle, and lower intestinal tract, including both the ceca and the rectum-cloaca area. The swabs should be deposited in 10 mL TBG broth, incubated, and plated as previously described for the internal organs. In addition, portions of the gut should be pooled, ground, or blended in 10 times their volume of TBG broth. Ten mL of the suspension from the digestive tract are transferred to 100 mL TBG broth and incubated at 42 or 37°C for 24 hours. The higher incubation temperatures for TBG broth reduce populations of competitive flora common in gut tissue.

Suspect colonies are transferred to triple sugar-iron (TSI) agar and lysine-iron (LI) agar and incubated at 37°C for 24 hours. Cultures revealing typical reactions of salmonellae or arizonae on TSI or LI agar slants should be identified by appropriate biochemical and other tests. All *Salmonella* cultures should be serologically typed.

Use of nonselective media demands careful aseptic techniques but has the advantage of more dependably ensuring the isolation of *S.* Pullorum and *S.* Gallinarum. Also, other bacteria capable of producing cross-reactions with pullorum-typhoid antigen may be more dependably isolated.

Identification of Cultures

The colonies of *S.* Pullorum may appear small, smooth, and translucent on nutrient media after 24 hours of incubation. With *S.* Gallinarum, colonies are smooth, blue-gray, moist, circular, and entire. Careful initial culture of tissues on nonselective media should usually result in pure cultures. If pure cultures are not secured, or if an enriched medium has been used, it is often advantageous to transfer individual colonies to TSI agar slants for preliminary differentiation. *S.* Pullorum and *S.* Gallinarum produce a red slant with a yellow butt that shows delayed blackening from H_2S production. Reactions listed in Table 16.1, which can be determined within 24 hours, provide identification of a number of other common pathogens and allow differentiation between the two organisms.

Additional differentiation tests described under Etiology may be necessary to identify isolates that produce nontypical reactions (chiefly fermentation of maltose or no gas production). Decarboxylation of ornithine by *S. pullorum* is the single most dependable test for differentiating maltose-fermenting *S.* Pullorum strains from *S.* Gallinarum. Polymerase chain reaction has also been used to identify isolates of *S.* Pullorum and *S.* Gallinarum (88, 89, 166).

Serology

Serologic tests to detect PD and FT include the macroscopic tube agglutination (TA) test, rapid serum (RS) test, stained antigen whole blood (WB) test, and the microagglutination (MA) test using tetrazolium-stained antigens (62, 86, 123, 131, 137, 153, 162, 179). The standard procedure in the United States for detecting breeding flocks chronically infected with *S.* Pullorum and *S.* Gallinarum is to use the standard strains of *S.* Pullorum (O–1, 9, 12_3) for tube and serum plate antigens and both standard (O–1, 9, 12_3) and variant (O–1, 9, 12_2) strains of *S.* Pullorum for the polyvalent rapid whole blood plate antigens. These antigens will detect flocks infected with either *S.* Pullorum or *S.* Gallinarum. Hens infected with antigenically intermediate or variant strains of *S.* Pullorum were detected as seropositive less often than were hens infected with antigenically standard strains with two commercially available plate test antigens (62).

Table 16.1. Biochemical reactions useful in differentiating *S. gallinarum* and *S. Pullorum.*

Reactant or characterstic	*S. gallinarum*	*S. pullorum*
Dextrose	Fermented with no gas	Fermented with gas
Lactose	Not fermented	Not fermented
Sucrose	Not fermented	Not fermented
Mannitol	Fermented with no gas	Fermented with gas
Maltose	Fermented with no gas	Usually not fermented
Dulcitol	Fermented with no gas	Not fermented
Ornithine	Not fermented	Fermented
Indole	Not produced	Not produced
Urea	Not hydrolyzed	Not hydrolyzed
Motility	Nonmotile	Nonmotile
Agglutination	Positive with group D	Positive with group D

The techniques and procedures for official testing of chicken and turkey breeding flocks and the interpretation of tests are described in detail in the latest version of the NPIP (10).

Enzyme-linked immunosorbent assays (ELISA) for detecting *S.* Pullorum and *S.* Gallinarum antibodies have been developed by using lipopolysaccharides or whole cell antigen from these salmonellae (16, 30, 113, 114). This technique can be used for screening large numbers of blood or egg yolk samples and can be adapted to differentiate vaccinated birds from those infected with wild-type strains (16, 113, 114). Infection with both serovars can also be differentiated serologically from *S.* Enteritidis infection using flagella as an antigen (16, 30, 64). Another test, a dot immunobinding assay (DIA), was found to be more sensitive compared to the tube agglutination test for detecting high titers of antibodies in birds challenged with *S.* Gallinarum (105). This test also detected antibodies to *S.* Gallinarum in vaccinated birds that failed to react by TA.

Differential Diagnosis

The clinical signs and lesions produced by PD or FT are not pathognomonic. Other *Salmonella* infections may produce similar lesions in the liver, spleen, and intestine, which cannot be distinguished grossly or microscopically from those produced by PD or FT. *Aspergillus* or other fungi may produce similar lesions in the lungs.

S. Pullorum and *S.* Gallinarum can localize in major joints and tendon sheaths of chicks. Such signs and lesions resemble those produced by organisms such as *Mycoplasma synoviae*, *Staphylococcus aureus*, *Pasteurella multocida*, or *Erysipelothrix rhusiopathiae.* Sometimes the white nodules in the heart of young chicks may resemble Marek's disease tumors and those in the

liver resemble those produced by *Yersinia pseudotuberculosis* with which it shares some antigenic relationships. Local infections with *S.* Pullorum and *S.* Gallinarum in adult carriers, particularly of the ovary, may appear identical to those produced by other bacterial infections such as coliforms, staphylococci, *P. multocida,* streptococci, and other salmonellae. Birds of any age may be infected with *S.* Pullorum or *S.* Gallinarum but fail to show grossly discernable lesions. A definitive diagnosis of PD and FT can be made only following the isolation and identification of *S.* Pullorum and *S.* Gallinarum, respectively.

Intervention Strategies

It has long been established that chicken and turkey flocks can be developed and maintained free of PD and FT by adhering to well-defined procedures. Both PD and FT are good examples of diseases that have decreased in incidence over the years by the application of basic management procedures. In the simplest sense, the only requirement is to establish breeding flocks free of *S.* Pullorum and *S.* Gallinarum and to hatch and rear their progeny under conditions that preclude direct or indirect contact with infected chickens or turkeys.

Management Procedures

Methods of management broadly designed to prevent the introduction of infectious agents are applicable to preventing the introduction of *S.* Pullorum and *S.* Gallinarum. Because of egg transmission, only eggs from flocks known to be free of PD and FT should be used in hatcheries. Under the NPIP, chicken and turkey breeding flocks and their progeny may be recognized as free of PD and FT.

Management practices should be broadly applied to prevent the introduction of PD or FT. If PD or FT is encountered, elimination of carriers must be carried out regularly until the breeder flocks are free of PD and FT.

1. Chicks and poults should be obtained from sources free of PD and FT.
2. Pullorum-free and typhoid-free stock should not be mixed with other poultry or confined birds.
3. Chicks and poults should be placed in an environment that can be cleaned and sanitized to eliminate any residential salmonellae from previous flocks (see "Susceptibility to Chemical and Physical Agents").
4. Chicks and poults should receive pelletized, crumbled feed to minimize the introduction of *S.* Pullorum and *S.* Gallinarum and other salmonellae through contaminated feed ingredients. Use of feed ingredients free of salmonellae is essential.
5. Introduction of salmonellae from outside sources must be minimized by the use of a sound biosecurity program.
   a. Free-flying birds are commonly found to be carriers of other salmonellae, although rarely with *S.* Pullorum or *S.* Gallinarum. Poultry houses should be bird proof.
   b. Rats, mice, rabbits, cats, dogs, and pests may be carriers of other salmonellae, but they are infected rarely with *S.* Pullorum or *S.* Gallinarum. Nevertheless, poultry houses should be vermin proof.
   c. Insect control is important, particularly against flies, poultry mites, and the lesser mealworm. These pests may provide a means of survival for salmonellae and other avian pathogens in the environment.
   d. Potable drinking water must be used, or chlorinated water should be provided. In some areas, a danger is posed by surface water collected in open ponds for use as drinking water for livestock and poultry.
   e. Mechanical carriers of the organism include footwear and clothing of humans, as well as poultry equipment, processing trucks, and poultry crates. Every precaution should be made to prevent the introduction of *S.* Pullorum or *S.* Gallinarum by fomites.
   f. Proper disposal of dead birds is essential.

Elimination of Carriers

The foundation of the PD control program was established in 1913 by use of TA for detecting infected chickens (86). The test was promptly applied in state programs to eliminate the disease from flocks by the detection and removal of reactors.

Early field testing results indicated that removal of reactors following a single test usually was not sufficient for the complete elimination of infected birds from a flock. Such results may be expected because of three possible intercurrent characteristics: 1) serum agglutinin titers of infected birds tend to fluctuate and may for brief periods fail to produce significant agglutination at the usual dilution of 1:25 or 1:50; 2) a delay of at least several days exists between infection and the development of agglutinins; and 3) following the removal of reactors, environmental contamination may serve as a source of infection for other birds at a later date.

Serologic Tests

As noted previously, in addition to the TA test, other serologic tests such as the RS, WB, and MA tests have been developed (131, 137, 179). All of these are effective in detecting carriers. The MA test is as dependable as the TA test and offers an important advantage in lower cost. The NPIP (10), which details testing methods, accepts four tests for testing chickens: standard TA test, WB test, RS test, and MA test. Of the four, only the WB test is not satisfactory for turkeys. Testing for accreditation is allowed after chickens and turkeys reach approximate immunologic maturity at 16 weeks of age.

In contrast to requirements in the United States for producing antigen from cells grown on the surface of appropriate agar, a different WB test antigen was developed in Japan, where it is officially used (161). This antigen is prepared from cultures grown in a continuous-flow, broth-culture system, in which it is necessary to blend sublots to secure desired agglutinability. An ELISA test is also available for screening of flocks for PD and FT (16, 113, 114).

Serologic evidence of infection should be confirmed by bacteriologic examination of one or more reactors. If only suspicious reactions are observed in a flock, the birds reacting most strongly should be submitted to a laboratory for retesting and a thorough bacteriologic examination. In routine testing, flocks should not

be interpreted as infected solely on the basis of doubtful or atypical reactions, because such reactions may result from infections other than *S.* Pullorum or *S.* Gallinarum (63, 64, 144, 168).

Non-pullorum/Non-gallinarum Reactors

Non-pullorum, and possibly non-gallinarum, reactions occasionally cause problems in interpretation (60, 168). A variety of bacteria possessing antigens in common with, or closely related to, those of *S.* Pullorum may infect birds and produce an agglutinin response. It was reported that non-pullorum reactions occur more frequently with variant than with standard-form antigen. Infections with coliforms, micrococci, and streptococci, particularly those belonging to the Lancefield group D, were found to be responsible for a large percentage of non-pullorum reactions in chickens. Infections with other bacteria, such as *Staphylococcus epidermidis*, *Micrococcus* spp., *Aerobacter aerogenus*, *Y. pseudotuberculosis*, *Proteus* spp., *Escherichia coli,* and species of *arizonae, Providentia,* and *Citrobacter,* were responsible for many non-pullorum reactions. Other salmonellae, particularly those in group D, such as *S.* Enteritidis, may also produce cross-reactions. Non-pullorum reactors range from a few birds in a flock to as high as 30–40%. The character of the agglutination may be variable. Thorough bacteriologic examination of representative reactors is often the only dependable method of determining the infection status of a flock, and it is usually the only method of distinguishing between infections by *S.* Pullorum and *S.* Gallinarum.

National Control Program

The NPIP (10) details specific criteria for establishing and maintaining official U.S. pullorum-typhoid-clean flocks and hatcheries. These criteria are based on farm and hatchery management practices that prevent direct or indirect contact with infected stock and annual testing of all, or a representative portion, of the birds in the flocks.

If an attempt is made to free a flock of infection, retesting of the infected flock should be done at 2–4-week intervals until two consecutive negative tests of the entire flock are secured at not less than a 21-day interval. In the majority of cases, infection can be eliminated from the flock through short-interval testing. Two or three retests are often sufficient to detect all infected birds; occasionally, however, infection continues to spread within a flock. In some situations, the disease may not be eliminated through repeated testing.

Area Eradication

Here are the essentials of an eradication program for an area:

1. PD and FT must be mandatory reportable diseases.
2. Outbreaks must be placed under quarantine, and infected flocks marketed under supervision.
3. All reports of PD and FT must be investigated by an authorized state or federal official.
4. Importation regulations must require shipments of poultry and hatching eggs to be from sources considered free of PD and FT.
5. Regulations must require poultry going to public exhibition to be from flocks free from PD and FT.
6. Total participation of poultry breeding flocks and hatcheries must be required in a pullorum-typhoid control program, such as NPIP programs or the equivalent.

Forty-three states in the United States had qualified under the preceding program as pullorum-typhoid-clean states by 2000; however, a reservoir of PD still exists in small backyard and hobby flocks. This reservoir of infection may be larger than indicated, because not all states have a program to test noncommercial and exhibition poultry. Experience indicates that the usual separation of commercial and noncommercial poultry is quite effective in preventing the transmission of *S.* Pullorum and *S.* Gallinarum between these populations. Nevertheless, infected backyard flocks pose some danger to commercial flocks. It is necessary to continue to test commercial breeding flocks to enable earlier identification of accidental infections from noncommercial poultry.

Vaccination

Because PD has been mostly eradicated from commercial flocks over the years and the eradication program is in place, very little incentive exists for the production of vaccines to control PD. Fowl typhoid, however, continues to be a problem in some parts of the world. No federally licensed *S.* Gallinarum killed bacterin is produced in the United States, and live modified vaccines used in other countries are not permitted in the United States. Various investigators have evaluated killed and modified live vaccines as well as virulence plasmid-cured derivatives (11, 20, 28, 37, 69, 70, 71, 119, 147, 149, 187, 188). With the upsurge of FT in many countries, studies on the use of the 9R strain (149) as live oral or injectable vaccine, with or without oil adjuvants, have been reported with variable results (11, 69, 70, 71, 119, 147). Similarly, outer membrane proteins from *S.* Gallinarum have been reported to offer better protection than the 9R live vaccine in terms of clearance of the pathogenic strain from internal organs (30, 32). More recently, immunization against FT by the use of mutant strains of *S.* Gallinarum and a virulence plasmid-cured derivative of *S.* Gallinarum and other types appear to be promising in protecting birds challenged with *S.* Gallinarum (14, 15, 20, 67, 188).

Other Approaches

Incorporation of commercial formic acid preparations in the feed has been found to significantly reduce the experimental incidence and severity of FT (B29).

Competitive exclusion using gut flora preparations has also been reported to be effective against FT (118).

Treatment

Reasonably effective prophylactic and therapeutic drugs have been developed against PD and FT. In Canada and the United States, every effort has been made to eradicate these diseases so treatment is neither feasible nor desired.

Various sulfonamides, nitrofurans, chloramphenicol, tetracy-

clines, and aminoglycosides have been found to be effective in reducing mortality from PD and FT; however, no drug or combination of drugs has been found capable of eliminating infection from a treated flock. Sulfonamides, in particular, frequently suppress growth and may interfere with feed and water intake and egg production. Sulfonamides that have been used in the treatment of PD and FT include sulfadiazine, sulfamerazine, sulfathiazole, sulfamethazine, and sulfaquinoxaline (3, 34, 122).

Most of the studies have indicated, however, that appreciable numbers of infected birds remain among medicated survivors (29, 103). Spraying eggs with neomycin sulfate prior to incubation has also been helpful in controlling PD in chicks (157). Dipping contaminated eggs in antibiotic solution containing 400 ppm and 800 ppm of gentamicin was helpful in controlling *S.* Gallinarum in eggs (12). In addition, variable resistance to chlortetracycline and nitrofurazone has been reported among isolates of *S.* Pullorum (90, 135). Similar drug resistance to furazolidone (72, 150, 158, 159), to quinolones (99) and other antibiotics (100) by certain strains of *S.* Gallinarum has been reported.

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Paratyphoid Infections

Richard K. Gast

Introduction

The numerous motile members of the bacterial genus *Salmonella* are often referred to collectively as paratyphoid (PT) salmonellae. Found throughout the world, these organisms can infect a very wide variety of hosts (including invertebrate and vertebrate wildlife, domestic animals, and humans) to yield either asymptomatic intestinal carriage or clinical disease. First reported in avian species in 1895 in an outbreak of infectious enteritis in pigeons, PT infections have long been known to cause significant disease losses in young poultry. More recently, PT salmonellae have additionally been identified as important agents of foodborne human disease. Advances in poultry production practices, changes in consumer lifestyles and preferences, and heightened nutritional awareness have all combined to place poultry products among the leading sources of animal protein for much of the world. However, contaminated poultry meat and eggs have also consistently been among the most frequently implicated sources of human *Salmonella* outbreaks. Controlling PT infections in poultry flocks has thus become an important objective from both the economic and public health perspectives.

Economic Significance

Human illnesses resulting from the consumption of poultry products contaminated by *Salmonella* can be expensive for the poultry industry, governments, and affected individuals. The total combined costs of medical care, lost productivity, and premature deaths resulting from food-borne *Salmonella* infections of humans in the United States have been estimated to exceed $2 billion per year (142). Widely circulated media reports regarding *Salmonella* contamination of particular foods can significantly affect consumer demand for those items. International markets for poultry products are increasingly subject to restrictions based on food safety considerations.

Poultry producers also face many direct expenses from *Salmonella* infections in their flocks. Infections acquired vertically from parents or horizontally in the hatchery can cause significant growth depression or even mortality in young chicks or poults. Other diseases or stressful conditions can predispose mature poultry to severe *Salmonella* infections. Conversely, infection with *Salmonella* can increase the susceptibility of birds to other pathogens. Preventing the transmission of salmonellae to progeny or to humans can also be expensive for producers. The cost of risk reduction practices (including biosecurity, cleaning and disinfecting of facilities, rodent control, vaccination, and testing) for controlling *S.* Enteritidis infections in laying flocks in the United States has been estimated to be nearly 1 cent per dozen eggs produced (308).

Public Health Significance

Despite mounting concerns about other pathogens in recent years, *Salmonella* remains among the leading causes of foodborne disease throughout the world. According to the Centers for Disease Control and Prevention (CDC), salmonellae are responsible for at least 1.4 million illnesses, 15,000 hospitalizations, and 400 deaths each year in the United States (430). Widespread commercial distribution of contaminated foods can sometimes involve huge numbers of consumers in *Salmonella* outbreaks. For example, a 1994 *S.* Enteritidis outbreak associated with ice cream in the United States affected 224,000 people (200). *Salmonella* outbreaks can have particularly severe consequences for highly vulnerable populations in facilities such as day care centers and nursing homes.

Poultry products are frequently identified as important sources of salmonellae that cause human illness. An estimated 182,060 Americans became infected with *S. Enteritidis* during 2000 after consuming contaminated eggs (381). Approximately 80% of *S.* Enteritidis outbreaks occurring in the United States between 1985 and 1999 with an identified food source were attributed to eggs (340). Eating contaminated chicken has also been identified as a significant risk factor for *S.* Enteritidis infection (246). Illustrating the importance of poultry as a reservoir for the transmission of salmonellae to humans, many of the serotypes that are most prevalent in humans (such as *S.* Typhimurium and *S.* Enteritidis) are also found commonly in poultry (423).

Etiology

Classification and Nomenclature

The genus *Salmonella* is a member of the bacterial family Enterobacteriaceae and consists of five biochemically distinct subgenera (262). However, subdivision of this genus according to genetic relatedness yields only two species (178). One of these, *S. enterica*, includes more than 2500 motile and non-host-adapted PT serotypes such as *S. enterica* subspecies *enterica* serovar Enteritidis and *S. enterica* subspecies *enterica* serovar Typhimurium. The more traditional and succinct serotype desig-

nations (such as *S.* Enteritidis and *S.* Typhimurium) are still used to provide concise and convenient nomenclature for diagnostic classification and epidemiologic analysis.

Morphology and Staining

Salmonellae are straight, non-spore-forming rods, measuring about 0.7–1.5 × 2.0–5.0 μm. Salmonellae are Gram-negative, but cells can readily be stained with common dyes such as methylene blue or carbolfuchsin. PT salmonellae are usually peritrichously flagellated and motile, although naturally occurring nonmotile mutants are occasionally encountered.

Growth Requirements

Salmonellae are facultatively anaerobic and can grow well under both aerobic and anaerobic conditions. The optimum temperature to support *Salmonella* multiplication is 37°C, but some growth is observed over a range from about 5 to 45°C. Salmonellae can grow within a pH range of approximately 4.0 to 9.0, with an optimum pH around 7.0, although cellular components such as flagella and fimbriae may not be expressed under extreme pH conditions. The nutritional requirements of salmonellae are relatively simple, and most culture media that supply sources of carbon and nitrogen can support their growth. The viability of *Salmonella* cultures can be maintained for many years in simple media, such as peptone agar or nutrient agar, which have been stab-inoculated, sealed, and held at room temperature.

Colony Morphology

Typical *Salmonella* colonies on agar media are about 2 to 4 mm in diameter, round with smooth edges, slightly raised, and glistening.

Biochemical Properties

Typical PT salmonellae (211) ferment glucose (to produce both acid and gas), dulcitol, mannitol, maltose, and mucate, but do not ferment lactose, sucrose, malonate, or salicin. They can produce hydrogen sulfide on many types of media, decarboxylate ornithine and lysine, utilize citrate as a sole source of carbon, and reduce nitrates to nitrites. PT salmonellae do not hydrolyze urea or gelatin and do not produce indole.

Most PT salmonellae can be readily distinguished from the avian host-adapted serotypes, *S.* Pullorum and *S.* Gallinarum, on the basis of the inability of *S.* Pullorum strains to ferment mucate or dulcitol and the inability of *S.* Gallinarum strains to decarboxylate ornithine or produce gas from glucose fermentation. In addition, PT salmonellae are usually motile but *S.* Pullorum and *S.* Gallinarum are typically nonmotile. Strains of *S. enterica* subspecies *arizonae,* a clinically important pathogen of young turkeys, are differentiated from PT salmonellae by their ability to ferment malonate and their inability to ferment dulcitol.

Susceptibility to Chemical and Physical Agents

Physical Agents: Heat and Irradiation

Except for a few distinctively thermoresistant strains (such as *S.* Senftenberg 775W), salmonellae are generally susceptible to destruction by heat. Cooking poultry meat to an internal temperature of 74°C or higher will dependably destroy salmonellae (380). Heating at 57°C for at least 70 minutes can eliminate salmonellae inside intact eggs (42). Liquid whole egg is pasteurized in the United States according to USDA specifications that require a minimum treatment time of 3.5 min at 60°C (18). However, *Salmonella* can survive cooking methods for eggs that allow some of the yolk to remain liquid (115). Steam pelleting treatment of poultry feed under precisely defined conditions has been reported to kill salmonellae in a manner dependent on temperature, time, and moisture (204). Heating to at least 60°C (at 100% relative humidity) has been reported to provide an effective method for the decontamination of laying houses (177). The heat resistance of *Salmonella* can be increased by heat shock (440) or exposure to alkaline conditions (233), and decreased by refrigeration (373).

Irradiation has also been considered as an option for eliminating salmonellae from foods and feedstuffs. Most *Salmonella* strains appear to be highly susceptible to the lethal effects of irradiation (408). Gamma radiation has been successfully applied to reducing the levels of *Salmonella* contamination in poultry meat (325), egg products (291), shell eggs (401), and poultry feeds (274). Combined heat and radiation treatments have been shown to be more effective in destroying salmonellae than either treatment alone (409). Ultraviolet radiation has been found effective for reducing *Salmonella* contamination of poultry carcasses (432), hatching eggs (11), shell eggs (143), and egg belts (143).

Chemical Disinfectants

Diverse chemical treatments have shown efficacy for reducing the levels of contaminating salmonellae on poultry carcasses and eggs and in poultry facilities. However, recontamination of surfaces after disinfection can sometimes diminish the potential benefits of chemical treatments (37). Moreover, sublethal chemical treatment has been reported to induce bacterial thermotolerance (374). Hydrogen peroxide (313), acetic acid (122), lactic acid (237), potassium sorbate (310), chlorine (310), and trisodium phosphate (41) have all been reported to lower the incidence or level of *Salmonella* contamination on broiler carcasses. Fumigating with formaldehyde (445), hydrogen peroxide (11), or ozone (11), spraying with polyhexamethylene biguanide hydrochloride (87), or dipping in hydrogen peroxide (92), lactic acid (92), or a peroxidase catalyzed compound (264) have all been found to be useful for controlling salmonellae on hatching eggs. Standard commercial application of chlorine-based detergent-sanitizer compounds has demonstrated efficacy against salmonellae on shell eggs (318), as have iodine-based disinfectants (253), electrolyzed oxidizing water (38), and ozone (366).

Chemical disinfectants (especially phenolic and quaternary ammonium compounds) are also widely used in poultry housing facilities. However, cleaning and disinfection were only able to eliminate *S.* Enteritidis from 50% of contaminated laying houses during a large field trial in Pennsylvania (378). Disinfectants may not be effective against all strains of the same bacterial species (375) or against organisms in biofilms (356). The presence of chick fluff, feces, feed, or wood shavings can interfere with the

activity of many disinfectants (34). Some disinfectants also appear to have reduced potency when used with field (well, stream, or pond) sources of water (111). Chemical disinfection of poultry facilities can be compromised by the improper performance of cleaning and disinfection protocols or by recontamination of the environment by infected mice (107, 109). Formaldehyde fumigation can be highly effective for decontaminating poultry facilities (441), but safety considerations have limited its availability and use. Ozone fumigation has also been considered as a safer (although less effective) alternative (441).

Chemical treatment of poultry feedstuffs to inhibit salmonellae has also been evaluated. The inclusion of ethyl alcohol (189) or organic acids such as zinc acetate and zinc propionate (339) has been reported to significantly reduce *Salmonella* levels in experimentally inoculated feed. However, a study of 12 potential antagonists of salmonellae in poultry feed (including organic acids) found that only formalin was consistently effective (391).

Environmental Factors

The environmental persistence of PT salmonellae creates continuous opportunities for horizontal transmission of infection within and between flocks. *S.* Enteritidis has been observed to survive in litter and feed for 26 months after removal of an infected flock (103). However, used litter has sometimes been reported to exert an inhibitory effect on *Salmonella* growth or survival, perhaps because dissolved ammonia leads to a gradual increase in pH over time (415). The addition of lime was shown to increase the pH of poultry litter and reduce *Salmonella* survival (33). Moisture levels are an important supporting factor in allowing the persistence of salmonellae in poultry houses. The numbers of viable *Salmonella* detected in poultry litter have been found to be directly related to water activity levels (130) and accordingly tend to increase in regions of houses with reduced airflow (320).

Antigenic Structure

The traditional Kauffmann-White schema for antigenic classification of salmonellae is based on both somatic and flagellar antigens (133). The somatic "O" antigens are determined by polysaccharides associated with the body of the cell and are identified by arabic numerals. Serogroups (designated with uppercase letters) of salmonellae are defined by particular somatic antigens that are unique to members of the group. Most *Salmonella* isolates found in poultry belong to serogroups B, C, or D. The "H" antigens are determined by flagellar proteins and are usually identified by lower-case letters. Flagellar antigens sometimes occur in two different phases. The serotype of a particular *Salmonella* isolate is determined by the combination of O and H antigens that it expresses. Serotyping of isolates is generally accomplished using agglutination tests with batteries of specific antisera.

Strain Classification

Phage Typing

The differentiation of epidemiologically relevant strains within serotypes is often accomplished by determining their patterns of lysis with a defined set of bacteriophages. Phage typing has sometimes provided better discrimination between strains than antimicrobial susceptibility testing, plasmid analysis, ribotyping, or pulsed field gel electrophoresis (420). The various phage types of *S.* Enteritidis have provided the most widely used point of reference for establishing relationships between isolates from different sources (209). However, the dependability of phage typing is limited by the potential for the conversion of isolates to different phage types by mutation (349) or by the introduction of plasmids (44) or temperate phages (358).

Molecular and Antibiotic Susceptibility Typing

Numerous and diverse genetic analyses have been evaluated for their usefulness in improving the discrimination of epidemiologically relevant *Salmonella* isolates between and within serotypes and phage types. However, no single approach is demonstrably superior for all applications. The various available methods often differ in their relative utility for discrimination within particular serotypes (277). The most successful molecular typing methods for *Salmonella* isolates include pulsed field gel electrophoresis of chromosomal DNA (327), ribotyping (68), random amplification of polymorphic DNA (284), and plasmid profiling (276). The combined use of two or more typing methods frequently provides the most detailed differentiation of *Salmonella* strains (274). The pattern of resistance to antimicrobial agents (the antibiogram) has also been valuable as a *Salmonella* typing tool and is often used in conjunction with molecular analyses (327). These techniques have been reported to be capable of distinguishing between epidemiologically important outbreak strains of salmonellae and unrelated strains (65) and between strains from different geographic locations (226). They have also been used to link isolates obtained from diverse sources within an integrated commercial poultry enterprise (278) and to establish relationships between *Salmonella* isolates from poultry flocks and human disease outbreaks (316).

Virulence Factors

Toxins

Two general categories of toxins have been reported to play roles in the pathogenicity of PT salmonellae. Endotoxin is associated with the lipid A portion of *Salmonella* cell wall lipopolysaccharide (LPS). If released into the bloodstream of an infected animal when bacterial cells are lysed, endotoxin can produce fever. Intravenously administered *S.* Enteritidis endotoxin caused liver and spleen lesions in 2-week-old chickens (416). Lipopolysaccharide also contributes to the resistance of the bacterial cell wall to attack and digestion by host phagocytes. Loss of the ability to synthesize complete LPS has been associated with an impaired ability of *S.* Typhimurium to colonize the ceca and invade to the spleen in broiler chicks (96).

Several proteinaceous toxins have also been identified in *Salmonella*. Enterotoxin activity by salmonellae induces a secretory response by epithelial cells that results in fluid accumulation in the intestinal lumen (259). The heat-stable cytotoxin of salmonellae causes structural damage to intestinal epithelial cells, perhaps by inhibiting protein synthesis (257).

Adherence, Invasiveness, and Intracellular Survival

The adherence of PT salmonellae to intestinal epithelial cells is the pivotal first step in the sequence of events that produces disease. Strains of *Salmonella* with reduced ability to colonize the intestinal tract of chicks also have severely attenuated virulence (417). Both flagella and fimbriae of salmonellae have been extensively investigated as potential mediators of attachment. Mutants of *S.* Enteritidis lacking flagella were reported to exhibit reduced adherence to cultured avian intestinal cells (4) and did not compete effectively with wild-type strains to colonize the ceca of chicks (3). Similarly, *S.* Enteritidis strains lacking fimbriae were less often isolated from the ceca of inoculated chicks than were fimbriated strains (410). Glycosphingolipid and ganglioside receptors in the intestinal mucosa have been identified as relevant to fimbria-mediated attachment of *Salmonella* (273). However, some investigators have concluded that neither flagella nor fimbriae are entirely essential for *S.* Enteritidis colonization of the avian intestinal tract (121, 354). Lipoplysaccharide (O-antigen) has also been proposed to play a role in gastrointestinal attachment by salmonellae (54).

The overall virulence of salmonellae also depends heavily on the degree of mucosal invasiveness following adherence (5). Adherence and invasion appear to be separately regulated activities. Mutations that affected the intestinal colonization of chicks after oral infection with *S.* Enteritidis and *S.* Typhimurium did not affect virulence after intraperitoneal administration (348). Although adherence may not involve ongoing bacterial metabolic activity, the subsequent invasion of host cells requires protein synthesis by live salmonellae (286). The expression of some invasion-related bacterial proteins is evidently induced by contact with epithelial cell surfaces (463). Flagella and some types of fimbriae were found to play a role in *S.* Enteritidis invasion and dissemination to internal organs of chicks (121). Flagella-deficient (but not fimbria-deficient) mutants of *S.* Enteritidis were less able to invade to the livers and spleens of chicks (2). Type 1 fimbriae appear to mediate the colonization of tubular gland cells in the upper oviduct (112). However, other researchers were unable to identify any significant effect on the invasion of enterocytes, ingestion by macrophages, or virulence for chickens when fimbrial genes were inactive (354, 412).

Adherence and invasiveness of salmonellae can be influenced by culture growth conditions. Logarithmically growing *Salmonella* cells are more invasive in tissue culture than are cells in the stationary phase of growth, and salmonellae grown anaerobically have been shown to be both more adherent and more invasive than salmonellae grown aerobically (131, 269). Incubation with acetic acid increased (but incubation with propionic acid decreased) the subsequent invasiveness of salmonellae for chicken cecal epithelial cells (425). The infectivity of *Salmonella* cultures for chicks was lost fairly quickly during combined starvation and desiccation (271). The changing environmental conditions to which an enteric pathogen is exposed during the course of infection in an avian host may induce corresponding changes in the expression of virulence-related genes (127). For example, the high oxygen level and nutrient availability experienced in the gut might promote an invasive bacterial phenotype, but lower oxygen levels and nutrient availability after invasion might induce a different set of virulence proteins (185). Several characterized virulence genes are indeed apparently induced following invasion into cells (343). Different patterns of protein synthesis by *S.* Typhimurium have been observed within intestinal epithelial cells, macrophages, and liver cells (47).

The replication of salmonellae within host cells is also apparently necessary for the full expression of pathogenicity (272). Mutants of *S.* Typhimurium that were unable to survive within host macrophages (141) or to resist the antimicrobial effects of host peptides (179) were reported to exhibit reduced virulence. Both growth and killing of *Salmonella* seemingly occur simultaneously within macrophages (46). Salmonellae that survive after phagosome/lysosome fusion in the macrophage (330) may eventually destroy the macrophage itself (280). The production of iron-chelating siderophores may also contribute to the *in vivo* survival of salmonellae (459).

Plasmids

Plasmids are transmissible extrachromosomal DNA elements that have often been associated with bacterial pathogenicity. Serotype-specific plasmids of characteristic molecular weights have been directly linked with virulence for several salmonellae. Considerable homology has been demonstrated between virulence-associated plasmids of different serotypes (66). *Salmonella* strains cured of their virulence-associated plasmids have been found to be significantly less lethal for mice (198) and less persistent in the ceca of chicks (429). Plasmid-mediated virulence among *S.* Typhimurium and *S.* Enteritidis isolates has been variously associated with invasion of mesenteric lymph nodes, livers, and spleens (186), survival and multiplication in serum (62), intracellular growth (187), lysis of macrophages (184), and immunosuppression (207).

The pathogenicity of salmonellae, however, does not always require the presence of the serotype-specific plasmids. Some strains of *S.* Typhimurium, for example, have been shown to retain their invasiveness in cell culture assays (225) and their lethality for infected mice (337) in the absence of virulence-associated plasmids. Moreover, although a serotype-specific plasmid was found to be essential for the full expression of virulence by *S.* Enteritidis in mice, curing this plasmid did not affect *S.* Enteritidis colonization and invasion of the tissues of orally inoculated chickens (190).

Pathogenicity Differences of Strains, Serotypes, and Phage Types

Strains of PT salmonellae can differ greatly in their abilities to induce pathological effects in poultry. Significant disparities have been reported between *Salmonella* serotypes in the frequencies at which they cause mortality in chicks (370) or invade reproductive organs and contaminate eggs in mature hens (331). These virulence differences appear to be independent of the route of infection (301). However, lethality for chicks can also vary tremendously within single *Salmonella* serotypes, sometimes even among strains of the same phage type (25). Pathogenicity

differences between the various phage types of *S.* Enteritidis have been extensively investigated, with phage type 4 sometimes associated with a particularly high level of invasiveness (20) and lethality (152) for newly hatched chicks. However, in experimental infection studies with chickens, phage type 4 strains yielded similar frequencies of intestinal colonization, invasion to spleens, horizontal transmission, and egg contamination as isolates of other phage types (153, 160). Considerable dissimilarity in metabolic properties has been found to exist within as well as between *S.* Enteritidis phage types (307). Differences between strains of *S.* Enteritidis, crossing phage-type boundaries, have been noted in both virulence for chicks (152) and egg contamination by hens (387). Even within the same clonal genomic lineage, *S.* Enteritidis strains may not have the same virulence properties (335).

Several bacterial characteristics have been identified as contributing to the observed pathogenicity differences between *Salmonella* strains. Although some virulence genes are distributed rather widely in *Salmonella* isolates from diverse sources (401), an analysis of genes induced *in vivo* indicated that regions associated with some virulence attributes have accumulated differentially in individual strains (71). Properties including heat and acid tolerance (236), motility (270), mannose-sensitive hemagglutination (270), and the ability to invade and survive inside cultured cells (328) have been linked to the virulence of *Salmonella* isolates. Invasive properties of *S.* Enteritidis variants have been found to depend on quantitative and qualitative differences in LPS expression (342). The virulence of *S.* Enteritidis isolates for chicks and the frequency of deposition of these strains in eggs laid by infected hens have been associated with both the production of large amounts of high-molecular-weight LPS and with the ability to grow to unusually high cell densities in broth cultures (180, 181). The expression of some *Salmonella* virulence properties appears to be inducible *in vivo*, as strains reisolated from infected laying hens have displayed an increased ability to cause egg contamination (155).

Pathobiology and Epidemiology

Incidence of Salmonellae in Poultry and Poultry Products

Estimates of the incidence of *Salmonella* in meat-type poultry and poultry house environments have varied considerably. For example, surveys of turkey flocks have reported the isolation of salmonellae from 79% of litter samples and 70% of fecal samples in one study (376) and from 13% of litter samples and 11% of cecal samples in another (326). Likewise, surveys of the incidence of *Salmonella* infection in broiler flocks have yielded 39% positive results in one study (52) and 5.5% in another (435). *Salmonella* isolation rates can change considerably from year to year for flocks in a single country (134). The actual prevalence of infection or contamination within *Salmonella*-positive flocks can also vary widely, but appears to usually be relatively low (341). Investigators in the USA reported that the frequency of *Salmonella* isolation from broiler breeder hatcheries increased from 11% to 16% between 1991 and 1998, but the proportion of samples that were heavily contaminated declined from 36% to 4% over the same time period (93).

Surveys of egg-type poultry have similarly generated diverse results, reporting the recovery of salmonellae from 72% of laying house environmental samples in one study (239) and from 53% of environment and fecal samples in another (345). In a national survey in the USA, 7.1% of commercial laying houses were positive for *S.* Enteritidis (144). The number of *Salmonella* found in individual environmental samples is typically relatively low, although somewhat higher levels can be evident at the beginning of egg-laying and molting (364). The distribution of salmonellae within contaminated laying houses is not necessarily uniform, as illustrated by work in the USA which reported that 10.5% of laying houses, but only 1.1% of individual cage rows within these houses, were positive for *S.* Enteritidis (56).

Surveys of poultry carcasses and meat products have provided dramatically divergent results for different countries in recent years. Intense pathogen reduction programs have led to significant reductions over time in the incidence of *Salmonella* contamination on broiler carcasses in some nations (418, 448). For example, only 5.7% of carcasses in Wales (299) and 10% of carcasses in Belgium (173) were found to be contaminated with salmonellae. On the other hand, *Salmonella* contamination continues to occur at very high frequencies in other countries, as demonstrated by reports that 40% of poultry meat samples from Mexico (460) and 57% of such samples from Thailand (338) were positive. The number of *Salmonella* cells found on contaminated poultry meat products has generally been relatively low. *Salmonella* counts of only about 32 cells (even after storage for 7 days) were observed in carcass rinses in one investigation (16). Another study (431) found only 1–30 *Salmonella* organisms per broiler carcass. Increasing incidences of *Salmonella* contamination at progressively later processing stages suggest cross-contamination of carcasses (53).

Contamination of eggs with salmonellae has also become an important issue since the mid 1980s. *Salmonella* contamination has been reported in 3% to 4% of table eggs in Italy and Brazil (48, 405) and in 19% of liquid egg samples in Japan (316). Field studies of commercial poultry in the USA observed *S.* Enteritidis egg contamination frequencies of less than 0.03% from environmentally positive flocks (250, 378). The U.S. Department of Agriculture estimated the overall national incidence of egg contamination with *S.* Enteritidis at approximately 0.005% (128).

Distribution of Salmonella *Serotypes*

Although more than 2500 serotypes of *Salmonella* have been identified, only about 10% of these have been isolated from poultry. Moreover, an even smaller subset of serotypes accounts for the vast majority of poultry *Salmonella* isolates. The distribution of *Salmonella* serotypes from poultry sources varies geographically and changes over time, although several serotypes are consistently found at a high incidence. Based on data from clinical and environmental isolates submitted to the U.S. Department of Agriculture National Veterinary Service Laboratory between July 2002 and June 2003, the most commonly identified PT serotypes were *S.* Heidelberg, *S.* Kentucky, *S.* Typhimurium, *S.*

Braenderup, and *S.* Enteritidis in chickens and *S.* Senftenberg, *S.* Heidelberg, *S.* Hadar, *S.* Muenster, and *S.* Kentucky in turkeys (140). The important epidemiological connection between the poultry and human reservoirs of salmonellae is sometimes evident in similarities in the distribution of serotypes reported from these sources. Of the 10 serotypes most often reported to the Centers for Disease Control and Prevention from human sources in the United States in 2003 (*S.* Typhimurium and *S.* Enteritidis were most common), 5 appeared on similar lists for either chickens or turkeys during approximately the same period (140). The implementation of intensified food safety regulations in Australia led to changes in the relative incidences of *Salmonella* serotypes, but a significant linkage still remained between the serotypes found in poultry and those associated with human illness (400).

Reports of the frequency of isolation of *Salmonella* serotypes from poultry sources around the world have yielded a wide range of results, although several serotypes appear to be of continuing international significance. The unique epidemiologic association of *S.* Enteritidis with disease transmission via contaminated eggs has made the prevalence of this serotype a topic of particular interest. *S.* Enteritidis has been the most common serotype found in surveys of eggs from Italy (48), chicken carcasses from Poland, Spain, and Korea (51, 67, 300), and chicken flocks in Turkey and the Netherlands (52, 423). Other serotypes that have been found to be most prevalent in individual situations include *S.* Heidelberg in broilers, broiler breeders, and turkeys in Canada (182), layers in the USA (247), and turkeys in Denmark (341); *S.* Typhimurium in broiler chickens in the USA (278); *S.* Infantis and *S.* Livingstone in Japanese poultry environments (316, 317); and *S.* Kentucky and *S.* Agona in poultry meat in the USA (283, 462).

Natural and Experimental Hosts

Paratyphoid Infections in Young Poultry

PT infections often have far different consequences for newly hatched poultry than for more mature birds. In very susceptible young chicks and poults, PT infection can sometimes lead to illness and death at high frequencies. Older birds are considerably less susceptible to the lethal effects of PT salmonellae and may experience intestinal colonization and even systemic dissemination without significant morbidity or mortality. The development of resistance to salmonellae in young birds has often been attributed to the acquisition of protective microflora that either compete with salmonellae for intestinal receptor sites or produce antagonistic factors that inhibit *Salmonella* growth (398). Accordingly, significantly more orally administered *S.* Typhimurium cells adhered in the ceca of 2-day-old chicks than in those of 3- to 7-day-old chicks (146). Such resistance may begin to develop as early as 36 hours after hatching (27).

PT infection has been established by oral, intracloacal, intratracheal, intraocular, navel, and aerosol administration to chicks (86). The usual outcomes of PT infections in chicks and poults involve three stages. Orally introduced PT salmonellae first establish intestinal colonization, often resulting in persistent shedding in the feces. Naturally occurring, horizontal transmission of salmonellae may involve a slower course of infection than is typically seen in oral inoculation experiments (312). Second, invasion beyond the gastrointestinal tract can lead to *Salmonella* multiplication in the macrophage-phagocyte system of the liver and spleen (25) and eventual dissemination to colonize a variety of internal tissue sites. Third, extensive bacteremia sometimes occurs, occasionally causing high mortality. The incidence of both mortality (136) and intestinal colonization (372) in chicks correlates strongly with the dose of orally administered salmonellae.

Mortality associated with naturally occurring PT infections in poultry is often observed to reach peak levels at about 3 to 7 days of age (309). Studies of experimental PT infections in young poultry have consistently shown that newly hatched birds are highly susceptible to salmonellae, but this susceptibility decreases over time. For example, oral doses of 10^9 *S.* Typhimurium cells were lethal for 50% of 1-day-old broiler chicks, 20% of 3-day-old chicks, and no 7-day-old chicks (136). Age-associated declines in mortality have also been noted for infections with *S.* Enteritidis and *S.* Hadar in chicks (118, 123) and *S.* Typhimurium in turkey poults (39).

The frequency of both intestinal colonization (372) and invasion to internal organs (118) is higher in newly hatched chicks than in older birds. The persistence of salmonellae in various colonization sites is also influenced by the age of the birds when infected (123). Persistent gut colonization was observed after infection of chicks with various *Salmonella* serotypes within 2 days of hatching, but not when infected at 3 weeks (27). Horizontal contact exposure of chicks within 24 hours of hatching has been reported to result in fecal shedding of *S.* Enteritidis for at least 28 weeks (321). Intestinal persistence of *S.* Typhimurium and *S.* Enteritidis was far greater following oral inoculation of chicks at 1 day of age than at 7 days (35, 146). Administration of 10^2 *S.* Enteritidis cells to one-day-old chicks led to a more persistent intestinal infection than administration of 10^9 cell to one-week-old birds (426). After oral inoculation of chicks at 1 day of age, *S.* Enteritidis persisted in the intestinal tract and was shed in the feces of almost half of these birds at 24 weeks of age (158). Other investigators determined that a few chicks infected with *S.* Enteritidis at 2 days of age remained infected for up to 64 weeks (344). Age-related decreases in susceptibility to persistent *S.* Enteritidis infection have likewise been observed in the internal organs of orally inoculated chicks (116). Some internal organs have been reportedly positive for *Salmonella* as long as one year after inoculation of chicks at one day of age (389).

Paratyphoid Infections in Mature Poultry

Morbidity and mortality are not consistently associated with PT infections in mature poultry. Experimental infections of adult chickens with large oral doses of PT salmonellae have often been reported to cause no evident signs of clinical illness (228). Although oral inoculation of laying hens with *S.* Enteritidis resulted in frequent bacteremia and extensive systemic dissemination to internal organ sites, the inoculated birds remained clinically normal except for some brief mild diarrhea (414). However, other investigators observed that six of ten 1-year-old hens died after oral inoculation with a phage type 4 *S.* Enteritidis isolate (232).

The two most consistently observed features of PT infections in mature poultry are intestinal colonization and systemic dissemination to internal organs. During approximately the first 2–4 weeks following experimental oral infection of chickens or turkeys, PT salmonellae can generally be isolated from the intestinal tracts and voided feces of a high percentage of inoculated birds (147). Although the incidence of intestinal colonization and fecal shedding steadily declines thereafter, some *S.* Enteritidis strains have been shown to persist in the intestinal tract of laying chickens for several months after oral inoculation (148, 387).

Gut colonization by PT salmonellae is usually followed by invasion through the intestinal epithelium and dissemination to diverse internal organ sites. Although various other serotypes (including *S.* Typhimurium, *S.* Heidelberg and *S.* Infantis) are also known to be invasive for poultry, the patterns and consequences of systemic dissemination have been documented most extensively for *S.* Enteritidis. After experimental oral inoculation of laying hens, *S.* Enteritidis has been isolated from numerous internal tissues, including the liver, spleen, ovary, oviduct, heart blood, and peritoneum (148, 414). Dissemination of *S.* Enteritidis to diverse internal organs, including the ovary and oviduct, has also been recorded following intravenous (156), intratracheal (322), conjunctival (229), intravaginal (305), or intracloacal (305) inoculation, exposure to contaminated aerosols (156), or insemination with contaminated semen (360). The isolation of *S.* Enteritidis from a wide range of internal organs has similarly been reported in naturally infected poultry (222).

Another aspect of infections of mature chickens with some PT salmonellae that is of particular concern from a public health perspective is the production of *Salmonella*-contaminated eggs. Investigations of laying flocks implicated as the sources of eggs that caused human disease outbreaks have sometimes detected *S.* Enteritidis isolates of the same phage types found in affected humans, often with identical plasmid or chromosomal DNA profiles, in environmental samples, tissue samples, and eggs (201). Internal contamination of eggs with *S.* Enteritidis is usually postulated to be a consequence of highly invasive behavior that leads to colonization of the ovary and oviduct (242). A few other PT serotypes including *S.* Heidelberg (154) and multiple antibiotic-resistant *S.* Typhimurium DT104 (444) have been deposited inside eggs laid by experimentally infected hens.

The reported incidence of *S.* Enteritidis contamination of eggs laid by commercial flocks has usually been extremely low. In studies of 17 naturally infected laying flocks in the United Kingdom, *S.* Enteritidis was found in the contents of less than 1% of the eggs sampled (230). In two Canadian layer flocks that yielded *S.* Enteritidis isolates from both environmental and tissue samples, less than 0.06% of the eggs sampled were contaminated (346). In the USA, 18 of 60 commercial flocks with *S.* Enteritidis in their environments produced contaminated eggs, at a prevalence of 0.0264% (201). Naturally contaminated eggs have generally been found to contain very small numbers of *S.* Enteritidis (235), but the *S.* Enteritidis population in eggs can expand to more dangerous levels if eggs are held at growth-promoting temperatures (151). Contamination of egg contents by *Salmonella* has also been demonstrated in experimentally infected laying hens (156, 388). After oral inoculation of hens, *S.* Enteritidis has been detected in both yolk and albumen, although typically at levels of only a few cells of the pathogen per ml of liquid egg contents (147, 160). Initial *Salmonella* deposition is more likely to occur in association with the albumen or vitelline membrane than with the interior contents of the yolk (161).

Predisposing Factors

A number of factors have been demonstrated to increase the likelihood or severity of PT infection in poultry. Several other infectious agents have been reported to influence the course of infection with salmonellae. Prior infection with coccidia such as *Eimeria tenella* can increase the ability of diverse *Salmonella* serotypes to colonize the intestinal tracts of chickens (351). Exposure to *E. tenella* caused recrudescence of a previous *S.* Enteritidis infection in chickens (352). Decreased levels of *Salmonella*-inhibiting volatile fatty acids and an increased oxidation-reduction potential in the intestine may follow coccidial infection (8). Infection with *E. tenella*, however, was observed to decrease the frequency at which subsequently administered *S.* Enteritidis invaded to the internal organs of chicks, possibly by increasing the thickness of the intestinal lamina propria (406). Prior coccidial infection did not affect organ invasion or the production of internally contaminated eggs by hens inoculated with *S.* Enteritidis (353). Infections of poultry with immunosuppressive viruses or bacteria can also affect the outcome of *Salmonella* infections. Exposure to reticuloendotheliosis virus at 1 day of age increased mortality among chicks inoculated intraperitoneally with *S.* Typhimurium at 1, 7, or 14 days of age (311). Exposure of 1-day-old chicks to infectious bursal disease virus was associated with increased mortality following subsequent *S.* Typhimurium infection (457) and increased gross lesions following inoculation with *S.* Enteritidis (344). Suppression of cell-mediated immunity by *Corynebacterium parvum* led to increased morbidity in chicks subsequently infected with *S.* Typhimurium (85).

Environmental and management factors can also influence the susceptibility of poultry to PT salmonellae. Stressful conditions have often been shown to facilitate or exacerbate *Salmonella* infections. Overall flock health, manure management, ventilation, and watering systems have all been identified as relevant risk factors for *S.* Enteritidis infection in commercial laying flocks in the USA (56). Housing inoculated chicks at a high density in unsanitary conditions was reported to increase cecal carriage of *S.* Enteritidis (7). Lowering the brooding temperature of chicks by 5 to 8° C significantly increased mortality among newly hatched chicks inoculated with *S.* Worthington (407). Water deprivation before inoculation of 7-week-old chickens increased the duration of fecal shedding of orally administered *S.* Typhimurium (45). Feed withdrawal from broiler chickens before slaughter has been linked to increased *Salmonella* contamination of crops (77). In experimental infection studies with *S.* Enteritidis, the induction of molting in laying hens by feed deprivation was reported to increase the incidence and level of fecal shedding (214), the incidence and severity of intestinal lesions (347), the numbers and extent of distribution of *S.* Enteritidis within the intestinal tract

(219), invasion to livers and spleens (219), and horizontal (213) or airborne (220) transmission of infection. Molting also reduced the infectious dose of *S.* Enteritidis necessary to establish intestinal colonization in hens (212) and increased the likelihood of recurrence of previous *S.* Enteritidis infections (221). Feed deprivation appears to lower crop levels of protective lactobacilli and volatile fatty acids while increasing pH (126). A national study in the USA identified induced molting as a significant risk factor for the likelihood of laying flocks being infected with *S.* Enteritidis (144). Recent studies have shown that molting can be induced via alternative diets, including wheat middlings and alfalfa, without causing increased susceptibility to *Salmonella* (382, 452).

Sources, Vectors, and Transmission

PT salmonellae can be introduced into poultry flocks from many different sources. Feeds have often been identified as likely sources of *Salmonella* because of contaminated animal proteins and other ingredients (369). Dust in feed mills is another potential source of introduction of salmonellae into finished feeds (238). Contamination by salmonellae has been reported in 58% of finished feed (mash) samples and 92% of meat and bone meal samples (91). Meal and mash feeds are more often implicated as sources of *Salmonella* than are pelleted feeds (91, 369). The serotypes of salmonellae isolated from live poultry and carcasses have sometimes (but not always) been correlated with the serotypes found in feedstuffs (428, 461). A Japanese study reported similar chromosomal DNA patterns for *S.* Enteritidis isolates obtained from feed given to a commercial laying flock and the contents of eggs laid by these birds (386). Experimental inoculation studies have demonstrated that chicks can be readily infected by very low levels of PT salmonellae in their feed (206). Salmonellae have survived for two years in artificially inoculated feeds (108).

The extremely wide host range of PT salmonellae creates an equally large number of reservoirs of infectious organisms. Biologic vectors can both disseminate and amplify salmonellae in poultry flocks. In a study of 10 egg-laying farms in the United Kingdom, molecular fingerprinting linked *S.* Enteritidis isolates from wildlife vectors, the farm environment, mice, eggs, and hens (275). Insects and other invertebrates, including flies (334), litter or darkling beetles (390), ground beetles (103), cockroaches (258), and centipedes (103) can carry *Salmonella* organisms externally (and sometimes internally). Mice have been identified as particularly important vectors for *S.* Enteritidis in laying flocks (378). A large study of commercial laying flocks in the USA indicated that the prevalence of *S.* Enteritidis in mice from environmentally positive poultry houses was nearly four times greater than from negative houses (144). A single mouse fecal pellet can contain 10^5 *S.* Enteritidis cells (202). Diverse wild birds can carry *Salmonella* infections (359) and contact with wild birds or their droppings has sometimes been identified as a risk factor for commercial poultry (97, 108). Humans can also be a source of salmonellae transmissible to poultry, as shown by a California sewage treatment plant that apparently spread infection to both wild animals and a commercial laying flock (249).

Vertical transmission of PT salmonellae to the progeny of infected breeder flocks can result in internal or external contamination of eggs. Egg shells are often contaminated with PT salmonellae by fecal contamination during oviposition. The penetration of salmonellae into or through the shell and shell membranes can result in direct transmission of infection to the developing embryo or can lead to exposure of the chick to infectious *Salmonella* organisms when the shell structure is disrupted during hatching. Some PT serotypes, particularly *S.* Enteritidis, can be deposited in the contents of eggs before oviposition (147, 242). The resulting transovarian transmission of infection to progeny is an important aspect of the epidemiology of *S.* Enteritidis in chickens. The same *Salmonella* serotypes responsible for mortality in naturally infected chicks and poults have often also been isolated from their parent flocks (263, 309). A survey of 10 farms in France led to the conclusion that the greatest contribution to the eventual distribution of *Salmonella* serotypes in broiler houses came from the chicks themselves and not from their environment (266).

Any PT salmonellae carried in or on eggs can be spread extensively in the hatcheries. As chicks or poults pip through egg shells, salmonellae are released into the air and circulated around hatching cabinets on contaminated fluff and other hatching debris. One study found *Salmonella* on 17% of egg shell samples and 21% of chick rinse samples obtained from commercial broiler hatcheries in the USA (13). Another study similarly isolated salmonellae (of 12 different serotypes) from more than 75% of samples of egg fragments, belting material, and paper pads from broiler hatcheries (89). Newly hatched birds, lacking protective intestinal microflora, are highly susceptible to intestinal colonization by salmonellae. Nearly 44% of chicks from uncontaminated eggs became infected with *S.* Typhimurium when hatched along with surface-contaminated eggs (55). The prevalence of *Salmonella* in broiler hatcheries has been found to be greater than at other production stages and the serotypes found in hatcheries have correlated with those isolated from finished carcasses (14).

PT salmonellae can also spread horizontally within and between flocks. Strains of 10 *Salmonella* serotypes spread rapidly from infected day-old chicks to penmates reared on litter (393). *S.* Enteritidis has been found in the feces and internal organs of uninoculated laying hens housed in cages adjacent to those of orally inoculated birds (147, 148). Contaminated poultry house environments are often implicated as leading sources of PT salmonellae (263). One investigation concluded that *Salmonella* serotypes present in broiler houses or introduced into houses by vectors during the rearing period were more likely to appear on processed carcasses than were serotypes originating in the hatchery (265). Studies in Dutch and Japanese laying flocks have likewise suggested that infection was more likely acquired from farm environments than from breeding stocks (421, 458).

Horizontal transmission can be mediated by mechanisms including direct bird-to-bird contact, ingestion of contaminated feces or litter, contaminated water, or personnel and equipment. *Salmonella* isolation from the environment of turkey poults was reported to reached peak levels by two weeks after the placement of infected birds in the house (223). Another study (108) reported that *S.* Enteritidis persisted for at least 1 year in dust in an empty

poultry house (even after cleaning and disinfection). In France, 70% of examined flocks had *Salmonella*-positive dust or litter samples (369). Air samples collected both inside and outside of five commercial laying facilities were positive for salmonellae (110). Airborne transmission of experimental *S.* Enteritidis infection has been demonstrated, perhaps mediated by contaminated dust (165). Negative air ionization has been proposed as a mechanism for reducing *Salmonella* transmission in poultry flocks by limiting the circulation of contaminated dust particles. In experimental settings, ionizers have reduced both airborne dust levels and the airborne transmission of *S.* Enteritidis infection to chickens (363).

Clinical Signs

PT infection of poultry is usually associated with disease only in very young birds. The contamination of eggs with salmonellae may lead to a high level of embryo mortality and the rapid death of newly hatched birds before clinical signs are observed. Morbidity and mortality can be high during the first 2 weeks of life, with significant body weight loss or growth retardation (120), but signs of disease are observed much less often in older birds. The course of illness is normally relatively brief in individual birds. Signs of severe PT infection in young poultry are generally similar to those observed in connection with other avian *Salmonella* infections (pullorum disease and fowl typhoid) and with other bacteria that can cause acute septicemia. Although clinical disease is not normally associated with PT infections in mature poultry, some *S.* Enteritidis strains have been found to cause anorexia, diarrhea, and reduced egg production in experimentally infected laying hens (147, 387).

Typical signs of PT infection in chicks and poults include progressive somnolence with closed eyes, drooping wings, and ruffled feathers. Anorexia and emaciation are common. Affected birds are often seen to shiver and huddle near heat sources. Profuse watery diarrhea is frequently observed, often resulting in dehydration and pasting of the vent area. Blindness and lameness have occasionally been reported.

Pathology

In severe outbreaks of PT infection in newly hatched poultry, rapidly developing septicemia can cause a high incidence of mortality with few or no apparent lesions. When the course of disease is longer, severe enteritis is often accompanied by focal necrotic lesions in the mucosa of the small intestine. Cheesy cecal cores are sometimes observed. Spleens and livers are commonly swollen and congested, with evident hemorrhagic streaks or necrotic foci. Kidneys may also sometimes be enlarged and congested. Fibrinopurulent perihepatitis and pericarditis have been reported on numerous occasions. Slight inflammatory processes with heterophil infiltration ranging from focal to diffuse in distribution have been observed in the ovaries and oviducts of flocks naturally infected with *S.* Enteritidis (222). Unabsorbed, coagulated yolk material may be present in the yolk sac. Other lesions occasionally observed include peritonitis, yolk sac infection, pneumonia, hypopyon, panophthalmitis, purulent arthritis, serous typhilitis, airsacculitis, and omphalitis (370).

Pathogenesis of the Infectious Process

Although salmonellae can invade epithelial cells throughout the intestinal tract, the ceca and the ileocecal junction are often sites of particular affinity (416). After oral inoculation of day-old chicks, *S.* Enteritidis was observed to adhere to epithelial cells at the tips of villi (117). The invasion of intestinal epithelial cells by salmonellae leads to a series of pathologic changes that affect intestinal fluid and electrolyte regulation. This process can ultimately cause cell death and thereby produce and exacerbate diarrhea. Oral inoculation of laying hens with *S.* Enteritidis can produce inflammation of the epithelium and lamina propria of the colon and ceca related to heterophilic infiltration (199). In addition, epithelial invasion may also allow the removal of salmonellae through the basement membrane into the lamina propria by macrophages (43). *S.* Enteritidis was recovered from several internal organ sites of laying hens within as little as 1 hour after oral inoculation (231). The ability of salmonellae to survive and multiply in internal organs, particularly the liver and spleen, has been correlated with the comparative virulence of salmonellae in different host species (24). The spleen may provide a protected site where intracellular bacterial multiplication can continue without exposure to host defense mechanisms (125).

Immunity and Resistance

The immune response of poultry to PT salmonellae minimizes the duration and severity of infection and protects against reinfection. This response also permits the serologic detection of infected flocks and serves as the basis for efforts to protect birds against infection by vaccination. The development of immunity was demonstrated when oral reinfection of chickens with *S.* Typhimurium (10 weeks after the initial inoculation) resulted in reduced fecal shedding and more rapid clearance from tissues than was observed in previously uninfected birds (196). Administering immunosuppressive agents to chicks has been reported to increase mortality associated with PT infection (129). The progeny of immune laying hens can apparently acquire some degree of protective immunity (35). However, the development of protective immunity against further *Salmonella* challenges does not necessarily lead to the clearance of ongoing, persistent infection (399). Moreover, *Salmonella* infection of chickens can also cause lymphocyte depletion, atrophy of lymphoid organs, and immunosuppression, thereby facilitating the establishment of a persistent carrier state (193).

PT salmonellae can elicit strong antibody responses from infected poultry. Serologic positivity to *S.* Enteritidis and *S.* Typhimurium was reported to persist in different groups of infected chickens throughout a one-year study period (389). Experimental infection of chicks with *S.* Typhimurium induced strong IgG, IgA, and IgM responses in serum, intestinal contents, and bile which could be detected by antigens composed of whole bacterial cells, LPS, flagella, and outer-membrane proteins (196). When laying hens were orally infected with *S.* Enteritidis, serum antibodies were produced by most birds by 1 week postinoculation and reached peak values at 2 weeks postinoculation (149, 162). High serum IgG titers have been detected in laying hens for at least 27 weeks after experimental oral inoculation

with *S.* Enteritidis (26). In a naturally infected broiler breeder flock, 70% of the birds were found to be positive for serum antibodies to *S.* Enteritidis LPS at 35 weeks of age (76). Antibodies to *S.* Enteritidis have also been found in the yolks of eggs laid by infected hens. Specific antibodies were found as early as 9 days postinoculation and reached peak levels at 3–5 weeks postinoculation in eggs from hens experimentally infected with *S.* Enteritidis (150). Antibodies to *S.* Enteritidis have also been detected in eggs from naturally infected flocks (76).

Although less completely characterized than the antibody response, cell-mediated immunity to PT salmonellae has also been observed in poultry. A strong delayed hypersensitivity reaction was detected, using either whole bacterial cells or outer membrane proteins, between 2 and 5 weeks after experimental infection of chicks with *S.* Typhimurium (196). Heterophils of chickens and turkeys are strongly phagocytic and bactericidal for salmonellae (396) and apparently play a vital role in restricting organ invasion during the early phases of *S.* Enteritidis infection (256). The phagocytic and bactericidal activities of avian heterophils increase substantially during the first few weeks of life (439). Cytokines produced by sensitized T lymphocytes may play a particularly important role in conferring immunity on poultry, perhaps by expanding the pool of circulating phagocytic heterophils (254) and recruiting them to the site of infection (255).

The relative contributions of the antibody and cell-mediated responses in providing poultry with protective immunity against *Salmonella* infection have not been completely resolved. Strong antigen-specific cellular and humoral immune responses have both been temporally linked to clearance of *S.* Typhimurium infection in chickens (31). Likewise, a decline in the isolation of *S.* Enteritidis from reproductive tissues of laying hens during the second week of infection was associated with the proliferation of both T and B cells (450). Bursectomized chickens, deficient in the ability to mount an antibody response, were less able to clear *S.* Enteritidis infection from the intestinal tract and internal organs (119). Increased IgG and IgM levels in oviducts of experimentally infected hens were followed by partial clearance of *S.* Enteritidis (449). A group of hens infected with *S.* Enteritidis at 20 weeks of age produced high levels of IgM antibodies and showed no adverse signs, whereas a group of hens infected at 1 year of age produced much lower levels of antibodies and experienced significant mortality (232). However, other investigators have suggested that cellular responses are more critical for effective immunity (451). In one study, B cells were not found to play an essential role in the clearance of *S.* Typhimurium infection in chickens despite the induction of high antibody levels (29). In another study, intestinal clearance of experimental *S.* Enteritidis infection in chickens did not correlate with the musocal IgA response (218). Both the opsonic activity of specific antibodies and the phagocytic and lytic activity of cellular effectors may be necessary for the full expression of immunity (298). In addition to the antigen-specific adaptive immune responses, innate host phagocytic capabilities also contribute significantly to resistance against infection by salmonellae. Chicken macrophages have been reported to internalize higher numbers of *S.* Enteritidis cells and to clear intracellular salmonellae more rapidly than lymphocytes (260).

Genetically based differences in the innate resistance and immunity of lines of chickens to *Salmonella* infection have been widely discussed and studied in recent years. Chicks from distinct lines have been found to vary in their susceptibility to the lethal effects of *Salmonella* infection (183). Differences in the incidences of fecal shedding, organ invasion, and egg contamination have been observed between lines of mature chickens infected with *S.* Enteritidis (124, 174). Both innate and adaptive mechanisms have been proposed to explain differences in *Salmonella* susceptibility between lines of chickens. Macrophages from resistant lines showed greater and more rapid expression of pro-inflammatory cytokines and chemokines upon *Salmonella* challenge (443). Resistance to *S.* Typhimurium infection has also been correlated with an increased T cell response (30). However, although another study found reproducible significant differences between lines of chickens in the level and duration of fecal shedding of several *Salmonella* serotypes, no corresponding significant differences in antibody titers or circulating heterophil numbers were evident (22).

Diagnosis

Although clinical observations may suggest the likelihood of a PT infection, final diagnosis depends on the isolation and identification of causative organisms. Using conventional culture methods, this requires 48 to 96 hours (and even longer for some culturing protocols). A concise summary of traditional methods for isolating salmonellae from poultry was provided by Waltman *et al.* (434). Numerous faster alternative strategies for detecting and identifying salmonellae have also been proposed in recent years. Serologic detection of specific antibodies is often employed effectively as a rapid preliminary screening device to identify flocks that have been exposed to salmonellae.

Isolation and Identification of Causative Agent

Sample Selection

To identify PT infection in poultry flocks, samples are obtained and cultured from a variety of sources, principally including tissues, eggs, and the poultry house environment. The number of samples that must be processed to achieve a predetermined level of confidence of detection of PT infection in a flock is directly related to the size of the flock and inversely related to the actual prevalence of infection (1). In very large flocks estimated to have very low prevalences of *Salmonella* infection, samples from more than one bird are often pooled together before culturing to allow an adequate sample size to be attained within the limitations of existing laboratory resources.

As many PT *Salmonella* serotypes are highly invasive and can be systemically disseminated to numerous internal tissues, a diversity of different sites (including the liver, spleen, ovary, oviduct, testes, yolk sac, heart, heart blood, kidney, gallbladder, pancreas, synovia, and eye) can provide samples for diagnostic culturing. As lesions cannot be relied upon to indicate infected tissues, several different organs should be cultured from each bird (separately or together). As some highly invasive PT serotypes, particularly *S.* Enteritidis, can be deposited in the con-

tents of eggs before oviposition, culturing eggs has been applied as a test for assessing the potential threat to public health posed by infected laying flocks.

Because infections of poultry with PT salmonellae almost invariably involve colonization of the intestinal tract, samples of intestinal tissues and contents are frequently the focus of *Salmonella*-culturing efforts. In a survey of birds submitted to a diagnostic laboratory, salmonellae were found exclusively in intestinal samples in 78% of the chickens and 70% of the turkeys (135). In experimentally inoculated laying hens, *S.* Enteritidis was recovered more often from the intestinal tract than from any other tissue sampled (148). The caudal ileum, ceca, cecal tonsils, and cecal contents are the intestinal sites most often recommended for recovering salmonellae. Cloacal swabs or samples of voided feces have been used to provide evidence of persistent intestinal colonization by salmonellae in individual birds. The often intermittent pattern of shedding of salmonellae in the feces of infected birds tends to diminish the overall reliability of cloacal swabs for diagnosing infection (446).

Fecal shedding of salmonellae into the poultry house environment by infected birds makes culturing environmental samples a useful diagnostic tool. Moreover, environmental samples also provide an opportunity to monitor for the introduction of salmonellae into poultry houses by vectors, personnel, equipment, and other sources. Although sampling fresh feces themselves likely provides the most sensitive test for the shedding of salmonellae (203), sampling floor litter can sometimes provide a comparable level of detection (377). Experimental *S.* Typhimurium infection in laying flocks was detected more consistently over a period of 1 year by culturing floor litter than by any other testing approach evaluated (332). Drag-swab samples, obtained by dragging moistened gauze pads across the floor of poultry houses, have been reported to detect salmonellae with greater sensitivity than litter sampling (251). The use of multiple-swab assemblies can further improve the sensitivity of this method (50). Swabs dragged through wet areas of manure appear to be more productive than swabs from dry areas (368). Foot covers worn in poultry houses can also provide an effective sample for detecting environmental salmonellae (292). Nest boxes, egg belts, dropping belts or scrapers, fan blades, and dust have been identified as particularly productive sources for *Salmonella* sampling in commercial egg laying houses (100, 248). Dust can remain contaminated with salmonellae even after cleaning and disinfection of poultry houses (203). Air sampling has detected *Salmonella* in both hatching cabinets and rooms containing infected chickens (36, 166). Hatcher fluff is frequently contaminated with salmonellae, offering an opportunity for early detection of infection in flocks (304). Culturing poultry feed for salmonellae is often important in establishing the source of infection of a flock with a particular serotype (392).

Standard Culture Methods for Salmonella Detection

Although a very diverse assortment of culture conditions has been proposed for the isolation and identification of PT salmonellae, most standard methods follow a general scheme that involves four principal stages. First, nonselective pre-enrichment is used to encourage the growth of very small numbers of salmonellae or to allow the recovery of injured *Salmonella* cells. Pre-enrichment is not advisable when testing samples (such as intestinal contents or feces) with large numbers of competing organisms that might overgrow salmonellae in the nonselective broth. Second, selective enrichment is used to allow additional expansion of the *Salmonella* population while suppressing the growth of other organisms. Third, plating on selective agar media is used to obtain isolated colonies, each derived from a single cell. Nonselective agar plating media are also sometimes used with swabs from internal organs. Fourth, colonies with appearances characteristic of salmonellae are subjected to biochemical and serologic tests to confirm their genus and serotype identity. Virtually all proposed methods require the last two of these steps, but enrichment requirements vary according to the nature of the sample.

Tissue samples (except for samples of intestinal tissues or contents) from infected birds ordinarily contain relatively few competing organisms. Swab or loop samples taken from internal organs are often transferred directly to plates of both selective and nonselective agar media, without broth enrichment. Excised tissue samples, and any samples derived from the intestinal tract, are generally transferred initially into selective enrichment broth.

Because fecal contamination may result in the presence of diverse flora, eggshells are usually sampled for *Salmonella* without pre-enrichment (unless detecting the presence of other bacterial contaminants is also of interest). The surface of eggshells can be sampled by immersion or rinsing in broth media or the entire shell (including interior structures and shell membranes) can be sampled by aseptic breaking to release the contents followed by manual crushing and the addition of enrichment broth. In a study of commercially produced eggs, shell rinse and shell crush methodologies yielded similar frequencies of *Salmonella* recovery (319). Before culturing egg contents for contamination by salmonellae, the shell exterior must be disinfected to prevent fecal contaminants of the shell from being transferred to the contents during breaking.

Because of the very low prevalence of salmonellae (primarily *S.* Enteritidis) in egg contents, and because *Salmonella* contaminants tend to be present in eggs in very small numbers, the entire liquid contents of 10–20 eggs are often pooled together for sampling to minimize demands on laboratory resources. Egg contents pools are usually incubated before further culturing to allow the *Salmonella* population to expand to a consistently detectable level (157). Supplementation of whole egg pools with iron (63, 157) or concentrated broth enrichment media (159) can increase the multiplication of some *S.* Enteritidis strains during incubation. Pre-enrichment of egg contents has been shown to lead to a greater sensitivity of *S.* Enteritidis detection than direct selective enrichment (145), probably by allowing very small initial levels of salmonellae to expand to levels that will survive the harsher conditions of selective enrichment. Direct plating of incubated egg pools onto selective agar media can markedly reduce the time, media, and labor demands of culturing, but does so at a significant loss in detection sensitivity (145, 157).

Environmental samples are typically collected in sterile plastic

bags and subsequently cultured by transfer into selective enrichment broth. Moistened gauze pads can be used to sample environmental surfaces or can be dragged across floor litter or dropping pits. Transporting environmental drag-swab samples in double-strength skim milk is often used to support *Salmonella* detection, although good recovery has also been obtained using dry swabs. Feed should be tested by collecting several representative samples from each lot and transferring into selective enrichment broth. Pre-enrichment of poultry feed samples has been reported to be unnecessary or even counterproductive (90).

Culture media are generally incubated for 24 hours at 37° C. Longer (48-hour) incubation in nonselective media has been reported to be useful for recovering small numbers of *S.* Enteritidis from egg contents (147, 234). Shorter (6-hour) selective enrichment has been found inadequate to suppress competing microflora in heavily contaminated samples (99). Incubation of selective enrichment cultures at elevated temperatures (42–43° C) has been recommended to restrict the growth of competing organisms, especially in intestinal samples or samples containing fecal material (99). Delayed secondary enrichment, in which selective enrichment broth cultures are held for an additional 5 days at room temperature to allow salmonellae an extended opportunity to grow to detectable levels, has been found to improve the recovery of PT salmonellae from poultry diagnostic and environmental samples (434, 371).

Culture Media

A diverse assortment of media has been developed and recommended for isolating and identifying salmonellae. Although some evidence has suggested that proper selection of culture media is somewhat contingent upon the type of sample being tested, several commercially available formulations have been consistently effective for a variety of applications.

Suggested broth media for the pre-enrichment of samples for salmonellae include buffered peptone water and trypticase soy broth. The selective broth media most often used for isolating PT salmonellae in recent years are tetrathionate broth and Rappaport-Vassiliadis broth. Tetrathionate broth preparations have been found to yield a higher frequency of *Salmonella* detection than Rappaport-Vassiliadis broth or selenite-cystine broth from a variety of types of samples, including cloacal swabs, intestinal tissues, pooled egg contents, and poultry feeds (90, 145). Rappaport-Vassiliadis broth has been effectively used to isolate salmonellae from environmental samples and egg contents pools (234, 371). Concern about selenium toxicity for human laboratory workers has led to diminished use of selenite-cystine broth.

Numerous agar media are available for the isolation of PT salmonellae. Among the most commonly used plating media are brilliant green agar, XLD agar, XLT4 agar, bismuth sulfite agar, and Hektoen enteric agar. Brilliant green agar is perhaps the most widely used medium for *Salmonella* isolation from poultry sources and has been shown to be effective in application to diverse tissue, environmental, egg, feed, and air samples (166, 434). XLT4 agar has been successfully applied to detect salmonellae efficiently from poultry house environmental drag swabs (302). The addition of novobiocin to agar plating media has been demonstrated to improve *Salmonella* recovery by suppressing the growth of some competing organisms (notably *Proteus*) that might otherwise overgrow the salmonellae (404). Samples should always be streaked onto two different media, preferably with dissimilar indicator systems for differentiating salmonellae from other organisms.

Confirmation of Genus and Serotype

Colonies on selective agar plates that have the characteristic appearance of PT salmonellae must be tested further to confirm their genus identity and to determine their serotype. The combined use of triple sugar-iron agar and lysine-iron agar provides an effective presumptive test for identifying PT salmonellae. The observed pattern of fermentation with a battery of 6 carbohydrates can provide further differentiation of PT *Salmonella* isolates from other organisms (95). The serogroup of each isolate can be determined by agglutination tests with polyvalent antisera to groups of somatic O antigens, and the serotype can then be determined by slide agglutination tests with monovalent antisera to specific O antigens and tube agglutination tests with antisera to flagellar H antigens.

Rapid Detection Technologies

Obtaining negative results using conventional culturing methods for salmonellae requires several days for most types of samples, and confirming positive results adds even more time. A number of considerably faster techniques have become available and are steadily acquiring recognition for their usefulness in recent years, but these rapid methods have not yet supplanted traditional culturing for detecting *Salmonella* in poultry in most situations. Most of the rapid methods reduce the time requirements of testing by 1 or more days, and many are adaptable to some degree of automation. The principal concerns about rapid methods center around their typically high cost and poor sensitivity (commonly requiring at least one enrichment step to achieve a detectable cell density). For example, one study reported that small numbers of *S.* Enteritidis could not be consistently detected by rapid methods within a single working day (< 12 hours) of incubation for inoculated egg pools (163).

Although diverse other approaches have been applied with success, most efforts to develop rapid *Salmonella*-detection methods have centered around the use of specific antibodies or DNA probes. Specific antibodies to *Salmonella* antigens have been used to develop a variety of enzyme-linked immunosorbent assay (ELISA) methods. These tests, using polyclonal antibodies to *Salmonella* LPS or flagella, have been reported to detect salmonellae in eggs, tissues, cloacal swabs, environmental drag swabs, litter, and feed (197, 289). Monoclonal antibodies to outer membrane proteins or flagella have been used as the basis for ELISA tests to specifically detect *S.* Enteritidis in eggs, tissues, and environmental samples (241, 243). Although not apparently quite as sensitive as standard culture methods (402, 404), ELISA tests are usually reported to detect salmonellae at a frequency comparable to conventional culturing, and to do so at least 1 day sooner (456). One or more initial enrichment culturing steps, however, are generally necessary to allow the expansion of the

Salmonella population into the range detectable by ELISA, which is often estimated at between 10^5 and 10^7 salmonellae per ml (197, 241). Other antibody-based detection formats have demonstrated sensitivities similar to ELISA (164). Like conventional culturing methods, ELISA tests are also somewhat prone to false-positive results from competing flora able to grow in enrichment media (32).

Another application of antibodies for detecting salmonellae involves coating small magnetic beads with specific antibodies. When mixed with the sample to be tested, the antibody-coated beads will bind to any *Salmonella* target antigens present and a magnetic field can then be applied to recover the bead-antibody-antigen complex. In essence, immunomagnetic separation (IMS) thus serves as an alternative to broth enrichment for concentrating salmonellae, but with the advantages of requiring less time and having no adverse effect on sublethally injured cells (385). Concentration of salmonellae by IMS has also been reportedly more effective than by centrifugation (114). Using IMS to concentrate salmonellae before plating on selective agar has detected an equal or higher frequency of *Salmonella* contamination in samples of poultry meat, tissues, eggshells, feed, environmental samples, and cloacal or fecal swabs than did either traditional selective enrichment or motility-based enrichment (98, 285). IMS has also been used to detect small levels of *S.* Enteritidis contamination in pools of egg contents by both culturing and ELISA (216). IMS-based culturing removed up to 93% of *S.* Enteritidis from liquid eggs contaminated with 10^2–10^3 cells per ml (303) and detected <10 cells per ml in whole egg extracts (288).

An increasingly prominent approach to rapid testing for *Salmonella* in poultry involves using probes for particular DNA sequences unique to salmonellae or even to individual serotypes. Hybridization of the probe with DNA extracted from the sample indicates a positive result. The sensitivity of detection of salmonellae by DNA hybridization is similar to that of ELISA, and thus generally also requires one or more enrichment culturing steps (395). Moreover, some DNA hybridization assays are procedurally complex and are more expensive than most other available methods. The development of polymerase chain reaction (PCR) technology has allowed the specific amplification of particular target segments of DNA, thereby enabling hybridization reactions with probes to detect salmonellae in tissues, feces, environmental drag-swab samples, and eggs with a very high level of sensitivity (61, 384). Carefully chosen DNA probes can be used along with PCR to detect salmonellae with specific characteristics, such as those carrying genes for particular virulence properties (267), biochemical properties (279), or surface structures such as fimbriae (454). After appropriate enrichment culturing, PCR methods have detected initial contamination loads of <10 *Salmonella* cells in poultry environmental samples (282). PCR methods have similarly been capable of detecting very small numbers of salmonellae following the concentration of cells by IMS or centrifugation (294, 365).

Serologic Diagnosis of Infection

Specific antibodies to PT salmonellae have been found in the sera of both naturally (222, 427) and experimentally (21, 149) infected poultry with a high degree of sensitivity using diverse agglutination and enzyme immunoassay methods. Detectable serum antibody titers are often still present long after the clearance of all salmonellae from tissues and the cessation of fecal shedding (191). Because antibody tests only document prior exposure to salmonellae, and do not provide unequivocal evidence of a currently ongoing infection in a flock, positive serologic results must generally be followed by bacteriologic culturing for confirmation. Serology also generally provides positive results much later than bacteriologic culturing (240). Other problems with serologic testing include the possibility that subclinical infections will lead to fecal shedding without sufficient invasion and dissemination to elicit a detectable antibody response (332), the general immunologic unresponsiveness of very young birds to *Salmonella* infection (437), and cross-reactions between antibodies to similar PT serotypes (168).

Agglutination tests have detected both natural and experimental infections of chickens with PT salmonellae (149, 222). The principal agglutination assay formats include rapid whole-blood plate, serum plate, tube, and microwell plate tests. All of these tests rely on the ability of specific antibodies to cause visible agglutination when mixed with antigen preparations of killed whole *Salmonella* cells. Except for the tube test, all agglutination assays use stained antigens to improve the ease of visualization of the agglutination reaction. An additional incubation period with a secondary antibody (antiglobulin) directed against chicken immunoglobulins, by increasing the overall agglutination of the target antigen, has been reported to provide greater sensitivity for detecting PT infections than other agglutination test methods (72).

PT *Salmonella* infections in poultry have also been detected using numerous ELISA approaches. For example, ELISA tests with antigens including LPS, flagella, or outer membrane proteins have identified chickens infected naturally or experimentally with *S.* Typhimurium or *S.* Enteritidis (21, 245). An international collaborative effort reported a generally high degree of correspondence in the performance of a wide assortment of ELISA formats and antigens for detecting *S.* Enteritidis infections (23). By using very precisely defined antigens, ELISA tests often achieve a high degree of specificity and are thus frequently associated with fewer false-positive results due to cross-reactions between serotypes than are agglutination reactions (191, 245). Assays employing fimbrial antigens have shown a particularly high degree of specificity for identifying *S.* Enteritidis infections in chickens (355). The discriminatory potential of ELISA tests often depends on judicious selection of positive/negative cut-off values (293). Screening for serum antibodies using a flagella-based ELISA test has been applied successfully for controlling *S.* Enteritidis in Dutch breeder flocks (427).

Antibodies deposited in egg yolks can also be used to detect poultry infected with PT salmonellae. Both agglutination (150) and ELISA (167) tests have been applied to find antibodies to *S.* Enteritidis and *S.* Typhimurium in eggs from naturally and experimentally infected chickens. Egg yolk antibodies have been consistently detected by flagella-based ELISA in egg yolks from hens inoculated orally with as few as 10^3 cfu of *S.* Enteritidis (170). The detection of specific antibodies in eggs from commercial lay-

ing flocks in the USA was directly correlated with the presence of *S.* Enteritidis in tissue samples from those flocks (150). Likewise, a direct relationship was observed between specific egg yolk antibody titers and the incidence of shedding of *S.* Enteritidis in the feces of laying flocks in the Netherlands (422). Egg yolk antibody detection was slightly more effective than bacteriological culturing of voided feces for predicting *S.* Enteritidis contamination of eggs laid by experimentally infected hens (169).

Intervention Strategies

Risk Reduction and Testing

The diversity of sources from which salmonellae can be introduced into flocks or houses complicates efforts to establish specific critical control points for preventing PT infections in poultry (250). Effective prevention and control programs must involve coordinated and simultaneous implementation of risk reduction practices throughout the production continuum (224). Eggs and chicks (or poults) should be secured only from demonstrably *Salmonella*-free breeding flocks. Hatching eggs should be properly disinfected and hatched according to stringent sanitation standards. Poultry houses should be thoroughly cleaned and disinfected by recommended procedures between flocks. Rodent and insect control measures should be incorporated into house design and management and verified by periodic testing. Rigidly enforced biosecurity practices should be implemented to restrict movement of personnel and equipment onto poultry housing premises and between houses. Only pelleted feed or feed containing no animal protein should be used to minimize the likelihood of using contaminated rations. Water provided to poultry should come only from sources treated to ensure purity. Treatments such as medication, competitive exclusion cultures, or vaccination can be applied to reduce the susceptibility of birds to *Salmonella* infection. Finally, the *Salmonella* status of poultry and their environment should be monitored by frequent testing to verify the effectiveness of risk reduction practices. Such multifaceted prevention and control programs in individual states have been associated with a decreased incidence of *S.* Enteritidis infections in both egg-type chickens and humans in the USA (442, 314) and have accordingly been incorporated into a proposed federal regulatory plan (419). Significant progress in controlling salmonellae in poultry and poultry products has been attributed to similar national programs in a number of countries (287, 436).

Increased international interest in controlling *S.* Enteritidis in poultry has led to the development and implementation of numerous serotype-specific testing and monitoring programs in recent years. In the United States, the National Poultry Improvement Plan (NPIP) defines stringent sanitation and testing standards for breeder flocks to prevent the transmission of *S.* Enteritidis infection to egg-laying stock (362). Participation in this plan requires compliance with standards for feed selection and handling, disinfection of hatching eggs, and hatchery sanitation. NPIP testing for *S.* Enteritidis involves bacteriologic monitoring of the environment and serologic monitoring of birds, with culturing of tissues from selected birds used for confirmation. A proposed national *S.* Enteritidis testing protocol for laying flocks in the USA would screen for infection with drag-swab environmental samples and then confirm the threat posed to public health by culturing eggs (419).

Gastrointestinal Colonization Control

Newly hatched chicks and poults are highly susceptible to infection by PT salmonellae, but quickly become more resistant. This age-associated decrease in susceptibility to *Salmonella* is largely attributable to the acquisition of protective intestinal microflora from the environment. The ability of the normal bacterial flora of the gastrointestinal tract to inhibit colonization by salmonellae and other pathogens has served as the basis for the development of a diverse group of treatments often referred to collectively as competitive exclusion (CE). CE treatments involve administering defined or undefined bacterial cultures to poultry in order to diminish gastrointestinal colonization by enteric pathogens. Various nonmicrobial manipulations of gastrointestinal biochemistry have also been explored as colonization control options.

The efficacy of CE treatment has been illustrated repeatedly in both chickens and turkeys, using intestinal or fecal material from mature birds or undefined anaerobic cultures derived from such material. Administration of CE cultures has been shown to diminish both intestinal colonization and subsequent invasion to internal tissues by various PT salmonellae (329). The conditions under which CE preparations are obtained and handled can affect their efficacy. Administration of fresh turkey cecal material protected poults against *Salmonella* colonization better than day-old cecal material (208). Nevertheless, the protective efficacy of CE cultures can be maintained by continuous flow culturing (210). In field trials in commercial broiler chicken flocks, treatment with CE cultures has led to significant reductions in the incidence of salmonellae in live birds and on carcasses (17). CE administration to egg-type pullets before transfer into a contaminated laying house reduced the subsequent isolation of salmonellae from fecal and environmental samples (102). Treatment with CE cultures has sometimes been observed to enhance the clearance of concurrent or preexisting *Salmonella* infections (78). CE cultures have been shown to be effective against salmonellae following administration to poultry in a variety of forms, including crop gavage, application to the vent lip, whole-body spraying or droplet application, addition to drinking water, and encapsulation in lyophilized alginate beads added to the feed (80, 82). Protection has also been obtained by fogging CE cultures onto or injecting them into the air cell of hatching eggs (88, 350). Combined use of more than one route of administration has sometimes produced maximum protection (64).

Considerable research has sought to identify the microflora constituents responsible for protection against salmonellae. Specific individual microbial cultures including *Escherichia coli* (455), *Bacillus subtilis* (268), *Lactobacillus* species (188), *Bifidobacterium* species (139), and the yeast *Saccharomyces boulardii* (281) have been reported to exhibit protective (probiotic) activity against salmonellae in chickens. Some investigators have argued that a defined mixture of microorganisms can protect poultry with greater consistency than undefined cultures and

can also provide a greater assurance of safety than is available with mixtures of unknown organisms. The protective efficacy of mixtures of small numbers of intestinal bacteria is typically very limited (397), but more diversely defined mixtures can provide significant protection (84). Administration of a mixture of 29 defined bacterial cultures to broiler chicks significantly reduced colonization of the crops and ceca by *S.* Typhimurium (83). Used litter has also been used as a source of CE cultures (79).

The protective benefits of CE treatments have been attributed both to direct steric interference with the attachment of salmonellae to the intestinal epithelium and with inhibition of *Salmonella* growth in the intestinal tract as a consequence of lowered pH and increased levels of undissociated volatile fatty acids (379). Diverse feed additives have been investigated for their ability to either directly inhibit pathogen colonization or to support the growth of protective microflora. Adding various complex carbohydrates (including lactose, mannose, glucose, and fructooligosaccharide) to the feed or water of chickens has sometimes reduced crop or cecal colonization by salmonellae (59, 81). Dietary supplementation with formic, propionic, or caproic acids has also been associated with reduced isolation of *Salmonella* (411, 424). Administration of chlorate, lactic acid, or sucrose in the drinking water of broilers has been found to reduce the frequency of isolation of salmonellae from the crop after preslaughter feed withdrawal (49, 205).

Several factors have been identified that affect the overall usefulness of CE cultures for controlling PT salmonellae infections in poultry. Although CE treatment generally reduces the incidence of intestinal colonization by salmonellae, it does not prevent it altogether. Moreover, the protective efficacy of CE cultures can sometimes be overcome by severe *Salmonella* challenges (394). Because protection is most effective when CE cultures are given to chicks before they are exposed to pathogens, infection with salmonellae during hatching can compromise the protective value of CE treatment (12). Administration of CE cultures can thus contribute significantly to an overall *Salmonella* control effort, but proper cleaning and disinfection, biosecurity, rodent reduction, and other similar measures are still necessary to minimize the chances of exposure to salmonellae (336). Disruption of the normal intestinal microflora by antibiotic administration or feed and water deprivation can also interfere with the activities of CE cultures (10, 438).

Vaccination

Vaccination with either killed or live preparations has been found to reduce the susceptibility of poultry to PT infection. A decreased incidence of human *S.* Enteritidis infections in the United Kingdom followed the widespread vaccination of egg-laying hens (70). Vaccination of laying flocks has been observed to reduce the frequency of egg contamination with *S.* Enteritidis even when the pathogen is still detectable in the laying house environment (104). Live *Salmonella* vaccines have often been associated with a stronger or longer-lasting protective response in poultry, perhaps either because of adverse effects on relevant protective antigens during the preparation of killed vaccines or because live vaccines present relevant antigens to the host immune system more persistently (9). Killed vaccines may also fail to fully elicit the cell-mediated portion of the protective response (315). Nevertheless, both killed and live vaccines have been associated with significant protection against salmonellae, although neither type of vaccine has consistently provided an impenetrable barrier against infection, especially when high *Salmonella* challenge doses are involved (171, 367). Moreover, feed or water deprivation and environmental stresses such as heat may compromise the effectiveness of vaccines (323). Like competitive exclusion, vaccination is most effectively used as a component in a comprehensive program of risk reduction practices. Selecting or designing vaccines that provide protection but do not interfere with serological detection of infected birds has become a focus for research in recent years (306).

Interest in the use of killed vaccines (bacterins) in poultry has been renewed in the past two decades by escalating concerns about *S.* Enteritidis. Subcutaneous or intramuscular administration of adjuvanted bacterins to laying hens has been reported to reduce significantly the incidence of *S.* Enteritidis isolation from feces, internal tissues, and eggs following subsequent oral challenge (69, 171). Chickens vaccinated with bacterins have likewise been reported to exhibit reductions in mortality, lesions, clinical signs, organ invasion, and egg contamination for up to 12 weeks post-vaccination when challenged with *S.* Enteritidis by intravenous or intramuscular routes (413, 453). Bacterin administration to laying hens has moderated the increase in fecal shedding of *S.* Enteritidis that often follows induced molting (324). Field studies have associated bacterin administration with a reduced incidence of *S.* Enteritidis infection in Dutch broiler breeder flocks (137) and with consistently *S.* Enteritidis-negative fecal and environmental testing results from a British laying flock after vaccinated hens were transferred into previously contaminated facilities (101). Subunit vaccines composed of *Salmonella* outer-membrane proteins administered with adjuvants or incorporated into lipid-conjugated immunostimulating complexes have been efficacious against *S.* Enteritidis in chickens and turkeys (60, 244). Immunization of laying hens with purified *S.* Enteritidis fimbriae has provided protection against reproductive organ invasion and egg contamination (113).

Live attenuated vaccines need to persist in tissues long enough to induce a protective immune response, but should be avirulent and eventually cleared from vaccinated birds. PT *Salmonella* vaccine strains attenuated by several different approaches have been tested for their protective efficacy in poultry. Oral or intramuscular administration of various *aroA* mutants of *S.* Enteritidis (auxotrophs that do not grow well *in vivo* because of their inability to synthesize particular aromatic compounds) has reduced fecal shedding, horizontal transmission, organ invasion, and egg contamination after intravenous or aerosol challenge (73, 74). This protection has been found to persist for up to 23 weeks after administration of the vaccine strain (75). An orally administered δ*cya* δ*crp S.* Typhimurium strain (a double mutant with deletions of both adenylate cyclase and the cyclic AMP receptor protein) provided very strong protection against intestinal colonization and organ invasion by *S.* Typhimurium (192). A temperature-sensitive mutant (57) and a strain attenuated by repeated passage

in chicken heterophils (261) have also been shown to protect chickens against *S.* Enteritidis infection. Vaccination of hens with an avirulent *S.* Typhimurium strain was reported to reduced intestinal colonization of their progeny when challenged with virulent wild-type strains (194). A live vaccine was also useful for addressing the increase in susceptibility to *S.* Enteritidis infection associated with induced molting by feed deprivation (217). Evidence for cross-protection by live vaccine strains against other epidemiologically important *Salmonella* serotypes has been inconsistent. An avirulent *S.* Typhimurium vaccine reduced colonization, organ invasion, and egg contamination by *S.* Enteritidis (195), but *aroA- S.* Enteritidis strains did not cross-protect very effectively against *S.* Typhimurium challenge (74). The *S.* Gallinarum 9R vaccine strain has been evaluated extensively because it can generate protective immunity against *S.* Enteritidis without impairing serological detection of infected flocks (138). Even antigenically unrelated *Salmonella* strains have sometimes been observed to generate some degree of protection against subsequent challenge with *S.* Enteritidis, perhaps via a combination of immunological and competitive exclusion mechanisms (215). Several safety concerns about live *Salmonella* vaccines have also been raised, based on evidence that some vaccine strains may be genetically unstable (19) and can be detected for longer than anticipated in vaccinated hens if sufficiently sensitive culturing methods are used (403).

Prophylactic administration of lymphokines from immunized chickens has been shown to protect chicks against organ invasion after subsequent *S.* Enteritidis challenge (295). This effect appears to be due to increased phagocytosis and killing of *S.* Enteritidis by avian heterophils (117). Subcutaneous, oral, nasal, and *in ovo* administration of immune lymphokines to poultry have all provided some protection against *S.* Enteritidis challenge (252, 296), but this effect may be of relatively transient duration (172). Cross-protection against other *Salmonella* serotypes has also been observed (464).

Treatment

The efficacy and wisdom of medication with antibiotics to prevent or treat PT infections in poultry are topics of considerable debate. Antibiotics have a long history of widespread utilization in poultry at both therapeutic and subtherapeutic (growth-promoting) levels (388). Their usefulness for these purposes has been extensively documented in a variety of experimental and commercial settings. Antibiotics were employed effectively both as therapeutic and prophylactic agents as part of control efforts for *S.* Enteritidis in broiler and broiler breeder flocks in Northern Ireland (297). Preventive treatment of chickens with a variety of different antibiotics has been observed to prevent intestinal colonization of chicks by subsequently administered *S.* Enteritidis (58), and in some instances to clear pre-existing infections (176). Several antibiotics have likewise been reported to decrease fecal shedding of *Salmonella* when used as feed additives (40, 94). Provision of a competitive exclusion culture to restore a protective normal microflora after treatment with a fluoroquinolone antibiotic reduced fecal shedding of *S.* Enteritidis by broiler breeders (361), egg-type pullets, (105) and molted laying hens (383). *In ovo* administration of gentamicin was apparently effective against *Salmonella* infection without interfering with competitive exclusion treatment of the hatched chicks (15).

However, in the USA and in many other nations, current control practices for salmonellosis in poultry no longer regularly rely on antibiotics because of the inconsistent history of these drugs in eliminating *Salmonella* colonization and because indiscriminate veterinary and agricultural uses may imperil their medical usefulness by promoting microbial resistance (175, 388). Several investigators have documented limitations in the efficacy of antibiotics in controlling *Salmonella* in poultry. One study reported that five antimicrobial agents had very minimal value for preventing or eliminating experimental *S.* Typhimurium infection (333). Other investigators observed that feed additive antibiotics were associated with increased cecal *S.* Enteritidis numbers in chicks (7). Another study suggested that drug excretion by infected birds might have interfered with the recovery of salmonellae from cloacal swabs or feces and created an erroneous impression that treatment was effective (447). Combined administration of enrofloxacin and a competitive exclusion product reduced the incidence of *S.* Enteritidis carriage, but failed to eliminate infection from internal organs (227). The administration of some antibiotics has been reported to increase the susceptibility of poultry to *Salmonella* infection, perhaps by suppressing the growth of other microflora capable of exerting inhibitory activity against salmonellae (290). Discontinuing the use of antimicrobials for growth promotion in Denmark was followed by a decrease in the *Salmonella* prevalence in broilers (132). Both therapeutic and subtherapeutic antibiotic administration has been shown to select for drug-resistant strains of salmonellae (28, 357), thereby potentially compromising the effectiveness of those (and related) drugs in both animals and humans (106). Very high incidences of drug resistance have been reported among *Salmonella* isolates from both poultry production facilities and poultry products (6, 327), with a large proportion of these strains often displaying resistance to multiple antimicrobial agents (283).

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Arizonosis

H.L. Shivaprasad

Introduction

Arizonosis is a septicemic disease of young turkey poults caused by the bacterium *Salmonella enterica* subsp. *arizonae* (*S. arizonae*). Other species of birds such as chickens, ducks and wild birds, canaries and parrots are also susceptible. *S. arizonae* used to be one of the most frequently identified *Salmonella* serotypes in turkeys in the United States (30) and is related to significant morbidity and mortality. However, sporadic and serious outbreaks still occur through out the US (11, 31, 81. 96, 97). The disease is clinically indistinguishable from salmonellosis caused by other serotypes of salmonellae such as *S.* Typhimurium and *S.* Heidelberg.

S. arizonae represents an antigenically diverse group of bacteria (over 300 serotypes have been identified), which can be distinguished biochemically from other species in the genus *Salmonella.* Historically, the organisms now classified as *S. enterica arizonae* were included in the genus *Arizona,* and have commonly been referred to as the arizona group, arizonas, and paracolons. In 1982, the International Subcommittee on Taxonomy of Enterobacteriaceae decided that the arizona group should

be classed as two subspecies of the genus *Salmonella* based on the relatedness of their DNA with that of *Salmonella* spp. Earlier reviews on the arizona group and arizonosis have been published (3, 4, 46).

Definition and Synonyms

Arizona infection or avian arizonosis (AA) is an acute or chronic egg-transmitted disease of primarily of young turkey poults characterized by septicemia and neurological signs and blindness and increased mortality. Once disease caused by *Salmonella enterica arizonae* used to be called "paracolon" infection and members of the genus *Arizona* once were referred as "paracolons," *Arizona arizonae* and *Arizona hinshawii*.

Economic Significance

Avian arizonosis is of considerable economic significance to the turkey industry of North America and certain parts of the world through increased morbidity and mortality in young turkey poults and decreased egg production in turkey breeders and decreased hatchability (18, 19, 39, 51, 63, 94, 96, 100). The cost incurred in testing of carrier birds and eggs, supplies such as swabs, culling of birds, dipping of eggs and cleaning and disinfection, use of antibiotics for treatment of breeders and poults and labor adds substantially to the cost of eradicating the disease.

Public Health Significance

Infection in people due to *S. enterica arizonea* associated with birds and turkeys in particular has not been reported. However, arizonosis in humans associated with reptiles have been reported numerous times (8, 32, 60, 89, 103 and others).

History

Caldwell and Ryerson were the first to isolate the *Salmonella*-like organisms from diseased reptiles from the semiarid regions surrounding Tucson, Arizona (9). It is probable, however, that the bacteria were isolated earlier from poultry. Lewis and Hitchner (68) had previously reported recovery of slow lactose-fermenting bacteria from a disease of chicks resembling salmonellosis. This infection was probably due to a member of the arizona group, and may represent the first report of AA. In Great Britain, the first report of avian arizonosis in poultry was in 1968 (57).

Etiology

Classification

Since 1939, many attempts have been made to find a generally acceptable taxonomic position for this group of bacteria; several classification systems have been used and a wide variety of names and designations has been applied to the organisms.

Edwards and associates (cited in 72), established the biochemical and antigenic similarity of the arizonae and salmonellae. Enough differences were found between the groups, however, to justify classification of the arizonae in a separate genus. Kauffmann and Edwards (59) first employed the name *Arizona arizonae,* which was also used by Ewing (23) for members of the genus *Arizona* (genus II of the tribe Salmonellae). A new type species name *Arizona hinshawii* had been proposed by Ewing (25) to pay honor to the pioneering work of W.R. Hinshaw on AA in turkeys, reptiles, and other animals. Kauffmann (58) subsequently included the arizonae in his subgenus III of the genus *Salmonella,* designating them *S. enterica arizonae* and listing their antigenic formulas only in the simplified Kauffmann-White scheme. The arizonae have been classified in the *Salmonella* genus in the 9th edition of *Bergey's Manual* (7) and all organisms in the group are designated *Salmonella arizonae.*

Ewing and his colleagues (24, 27, 28, 29, 72) have further clarified the definition by which the biochemical and antigenic characteristics of members of the genus *Arizona* may be readily differentiated from other Enterobacteriaceae. The terminology used by Centers for Disease Control and Prevention will be followed in this subchapter, e.g., *S. enterica arizonae,* 18:Z4, Z32 (see Antigenic Structure).

Morphology and Staining

The *S. enterica arizonae* resemble other enteric organisms. They are Gram-negative nonsporogenic bacilli that are motile by peritrichous flagella.

Growth Requirements

Members can be readily cultivated on ordinary liquid and solid laboratory media, revealing an abundant growth similar to that of the salmonellae. Most cultures grow very well on *Salmonella-Shigella* and brilliant green (BG) agars, as well as other solid media recommended for isolation of salmonellae. On initial isolation, colonies usually resemble those of salmonellae but may develop an indicator change typical of lactose fermenters after incubation for several days or weeks. Rapid lactose-fermenting strains, rare in poultry, cannot be distinguished from normal coliforms, which are usually inhibited by these media. Routine use of bismuth sulfite plating medium was recommended (21, 46, 72) to aid in preliminary recognition of lactose-fermenting Arizona strains before they are possibly discarded as coliforms.

Colony Morphology

Colonies of *S. enterica arizonae* appear similar to other salmonellae and do not have any special characteristics that would distinguish them. On blood agar they appear as dull white with round, convex, butyrous colonies. On XLT4 media they appear round, convex to flat, butyrous colonies with a black center or completely black and on BG agar round, convex to flat, butyrous colonies pink to pinkish red in color.

Biochemical Properties

Cultures possessing the biochemical characteristics shown in Table 16.2 are almost invariably classifiable serologically as members of *S. enterica arizonae* (14, 26, 28).

Most isolates from poultry, unlike other salmonellae, ferment lactose usually within 7–10 days' incubation. Failure of cultures

Table 16.2. Typical biochemical characteristics of *S. enterica arizonae.*

Dextrose	Fermented with gas
Lactose	Fermented, as a rule, slowly or promptly
Sucrose	Not fermented, as a rule
Mannitol	Fermented with gas
Maltose	Fermented with gas
Dulcitol	Not fermented
Inositol	Not fermented
Indole	Not produced, as a rule
Methyl red	Positive
Voges-Proskauer	No reaction
Hydrogen sulfide	Positive
Urea	Not hydrolyzed
Gelatin	Liquified slowly
Potassium cyanide	Negative, as a rule
Nitrates	Reduced
Motility	Positive
Betagalactosidase	Positive
Decarboxylases	
Lysine	Positive
Arginine	Positive, usually delayed
Ornithine	Positive
Malonate	Positive
Phenylalanine deaminase	Negative

to ferment dulcitol and inositol or to use D-tartrate, their slow liquefaction of gelatin, and their positive reactions in sodium malonate and betagalactosidase are most useful in distinguishing them from other members of the *Salmonella* group.

Citrobacter

For purposes of classification and identification, the *S. enterica arizonae* must be differentiated not only from other salmonellae, but also from the antigenically related genus *Citrobacter* of the tribe Salmonellae. Members of this genus are not known to be pathogenic for poultry, but from a diagnostic standpoint they may be confused with *Salmonella* cultures on initial isolation from fecal specimens. The former Bethesda-Ballerup "paracolons" (*P. intermedium*) are included in the genus *Citrobacter* along with cultures previously classified as *Escherichia freundii.*

Resistance to Chemical and Physical Agents

Arizonae are readily destroyed by heat and common disinfectants, but have survived in contaminated water for 5 mo, in contaminated feed for 17 mo, in soil on turkey ranges for 6–7 mo, and for 5 to 25 or more wk on materials and utensils in poultry houses (2, 35, 62, 63, 86, 91). Resistance properties are very similar to those of salmonellae.

Antigenic Structure

S. enterica arizonae strains are related serologically to the salmonellae and other Enterobacteriaceae, and procedures for study and identification of their antigenic structure are identical to those for paratyphoid organisms. Thirty-four somatic (O) and 43 flagellar (H) antigens have been demonstrated.

The serotype nomenclature system used in designating members of the genus *Salmonella* has been applied to *S. enterica arizonae.* In writing antigenic formulas, commas are used to separate O antigen factors, a colon to distinguish the O and H antigens, commas to separate H antigenic factors within a single phase, and a hyphen or dash to separate the first phase from the second and the second from the third, etc. Thus, the monophasic type species would be designated *S. enterica arizonae* 18:Z4, Z32.

Evolution of the nomenclature for salmonellae has resulted in some confusion over the identification of strains. Two serotypes that were previously designated as 7:1,7,8 and 7:1,2,6 are now recognized as 18:Z4,Z32 and 18:Z4,Z23, respectively. Confusion also exists because, even though there are only 34 O and 43 H antigens, sometimes an isolate is designated as 65:Z52,Z53. The latter is the designation that conforms to the system recognized by the Centers for Disease Control and Prevention, and the World Health Organization.

Pathogenesis and Epidemiology

Incidence and Distribution

Avian arizonosis occurs worldwide wherever poultry is raised. At one time, *S. enterica arizonae* 18:Z4, Z32 was endemic in turkey flocks of North America. Very high rates of isolation were reported in California in 1968 and 1969, but decreased considerably by 1972 (73). However, a resurgence of AA in the form of serious outbreaks has occurred in California between 1999 and 2006 (11, 96). The most common serotype isolated was *S. enterica arizonae* 18:Z4, Z23 followed by 18:Z4, Z32 (11). *S. enterica arizonae* has been eliminated from the turkey industry in Great Britain (5, 57, 101).

Natural and Experimental Hosts

S. enterica arizonae recognize no host barriers and are widely distributed in nature in a variety of avian, mammalian, and reptilian species (10, 17, 18, 19, 72, 82, 95, 110, 111).

Among poultry, AA is most frequently encountered in turkeys. Greenfield (43) noted that AA in chickens does not appear to be economically important, although chickens are affected by AA both naturally and experimentally (17, 68, 99). Dougherty (12) isolated *S. enterica arizonae* from duck livers, revealing lesions very similar to those produced by paratyphoid infections.

For a review of AA as a human disease causing gastroenteritis and frequently more serious enteric fever and focal infections, see Guckian *et al.* (49), Johnson *et al.* (56), Kelly *et al.* (60), Martin *et al.* (72), Waterman *et al.* (103), Weiss *et al.* (104), and Williams and Hobbs (108).

Transmission

The transmission cycle of AA in poultry is identical with that established for motile salmonellae (see Paratyphoid Infections). Infected adult birds are frequently intestinal carriers and spreaders of *S. enterica arizonae* for long periods (16). Wild birds (75), rats and mice (39), and reptiles (50, 52) have been cited as com-

mon sources of the organisms for poultry flocks but reptiles are probably the primary source of *S. enterica arizonae*.

Intestinal infections have been reported (51, 93), and Adler and Rosenwald (1) reported that AA in adult turkeys is confined primarily to the intestinal tract.

Transmission of AA through eggs has been reported by many workers (6, 16, 17, 18, 19, 41, 51), and recovery of *S. enterica arizonae* from ovaries and oviducts of adult turkeys (33, 51, 94, 96) suggests that transovarian transmission can occur. This can result in eggs contaminated with the bacteria and as a consequence there is decreased hatchability and increased mortality during the first week in the poults hatched. Direct contamination of the ovary by systemic infection can follow ingestion of the organisms (64, 94, 98). Perek *et al.* (84) isolated *S. enterica arizonae* from semen of cockerels.

S. enterica arizonae from fecal contamination have a penetration pattern through the shell and shell membranes of chicken eggs very similar to that of *S.* Typhimurium when incubated at 37.2°C, resulting in frequent presence of the organisms in chicken and turkey eggs (16, 41, 94, 107). Fecal contamination may spread the infection from other animal species to poultry. Goetz (39) found an AA infection rate of 90% in rats and 50% in mice on the premises of a turkey farm where the infection was a problem in poults. Various types of wild birds, reptiles, and many common animal species can also infect poultry flocks.

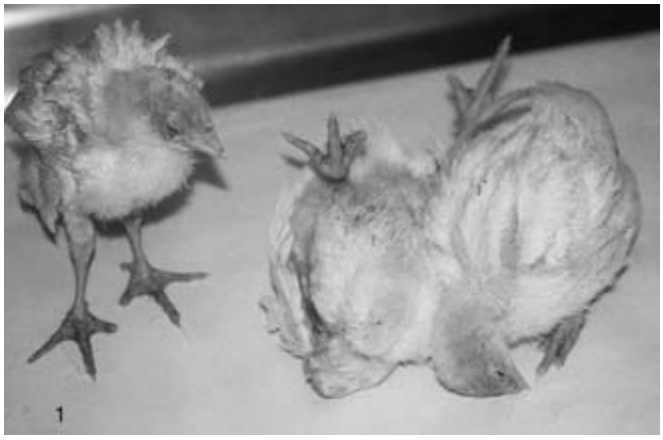
AA is transmitted in the incubator and brooder by direct contact and through contaminated feed and water (22, 63).

Clinical Signs

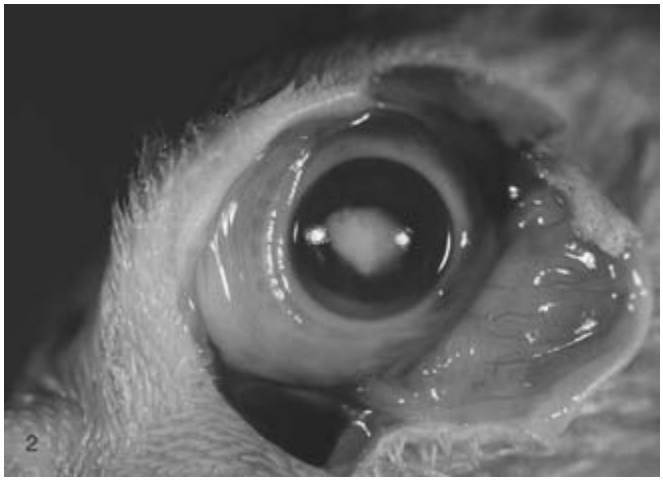
Although signs of AA in poultry are not specific, infected poults and chicks may appear listless, depressed and weak and develop anorexia, diarrhea, leg paralysis, and twisted necks (Fig. 16.5). There may be blindness due corneal opacity and/or caseous material in the anterior chamber and vitreous of the eye (Fig. 16.6) (10, 63, 93, 96, 99). Infected birds tend to sit on their hocks and huddle together. Nervous signs, including paralysis, torticollis, opisthotonus, convulsions, may follow brain and internal ear infection in poults (Fig. 16.5) (55, 82, 96). Sato and Adler (93) noted that clinical signs of AA are rarely seen in adult turkeys and they seldom die from this infection.

Mortality due to AA in poults and chicks is variable. Lewis and Hitchner (68) recorded mortality of 32–50% from the infection in chicks. In one study mortalities of 70 % by 7 days and 60% by 23 days after hatching have been reported (96). Mortality can be exacerbated by secondary or concurrent infections such as colibacillosis, poult enteritis, aspergillosis, coccidiosis, paratyphoid infections, crop mycosis, etc. (96).

Others have observed mortality generally between 10 and 50%, in chicks or poults especially susceptible within the 1st few days after hatching, and with mortality continuing for up to 3–5 wk (1, 17, 48, 63, 85, 96, 100). Geissler and Youssef (34) inoculated or dipped chicken eggs with *S. enterica arizonae*; 100% and 40–79% of embryos in the respective groups died during the incubation period. Hatchability for the latter group varied from 0 to 21–70%, with evidence that the organisms penetrated to the inner structures of the eggs.



16.5. Two-week-old turkey poults exhibiting depression and neurological signs due to arizonosis.



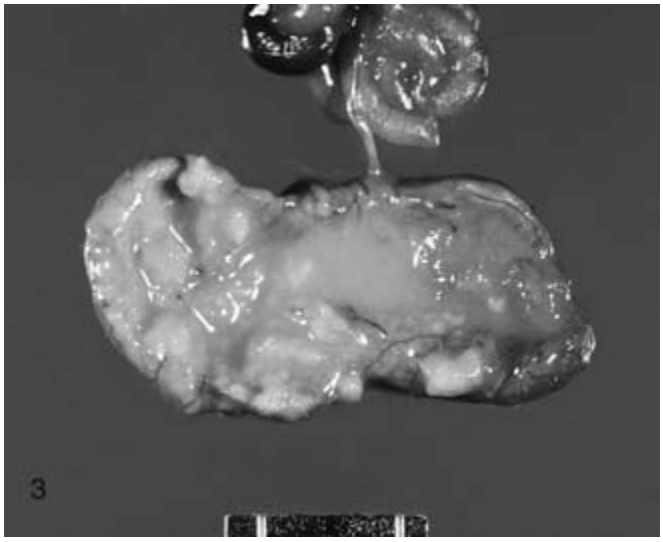
16.6. Eye of a three-week-old turkey poult with severe ophthalmitis.

Worcester (112) noted that *S. enterica arizonae* can penetrate the wall of the intestinal tract and stay there indefinitely.

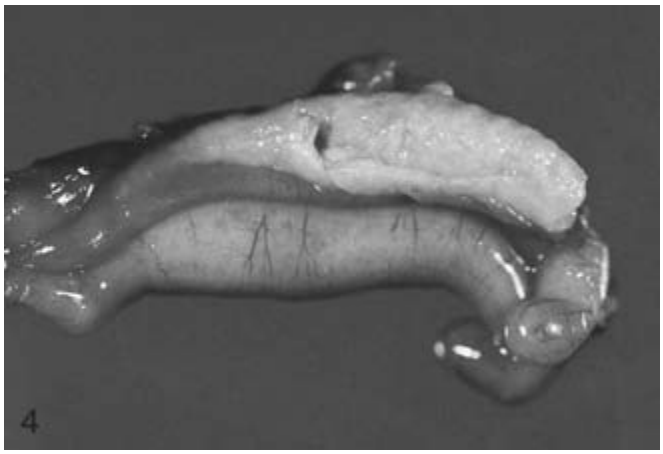
Pathology

Gross Lesions

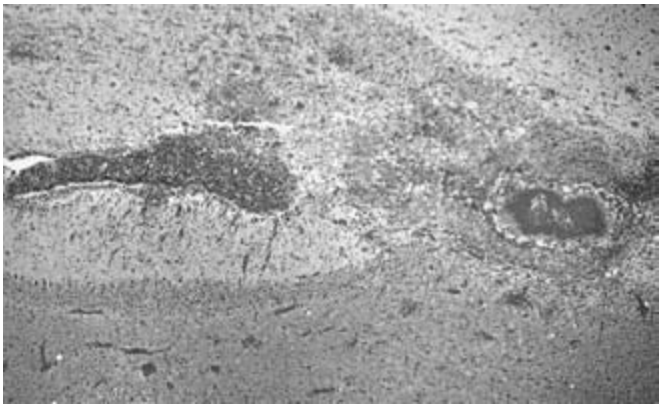
Gross and microscopic lesions due to AA in poults have been well described (11, 40, 90, 96, 99, 105). Lesions in poults, either naturally or experimentally infected with *S. enterica arizonae*, are comparable to lesions induced by paratyphoid organisms. In natural outbreaks there are retained yolk sacs, yolk sac with watery or caseous yellow contents (Fig. 16.7), prominent yolk stalks (navel buttons), cores in the ceca, and exudate on the meninges of the brain. Livers may be enlarged and mottled with white foci; there may be caseous exudate in the abdominal cavity, and discolored hearts. Pericardium and air sacs can be cloudy and there may be swollen joints (96). One of the most frequently noted pathologic changes in somewhat protracted cases is the presence of pale



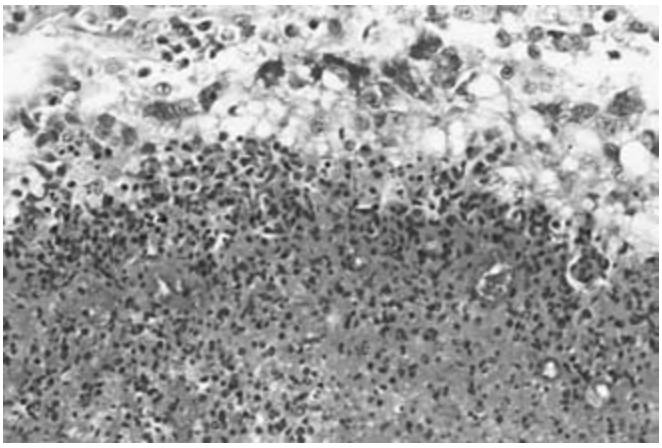
16.7. Severe yolk sac infection in a 5-day-old poult.



16.8. Ceca from a 5-day-old turkey poult with a core in the lumen.



16.9. Histopathology of brain with meningitis and encephalitis due to *Salmonella enterica arizonae* infection. ×300



16.10. Histopathology of internal ear with severe otitis and bacteria of *Salmonella enterica arizonae* infection. ×500

white or yellow exudate in the anterior chamber and vitreous of one or both eyeballs in poults (Fig. 16.6). However, lesions in the eye and brain develop in slightly later stages of the disease.

Lesions typical of generalized septicemia, including peritonitis, retained yolk sacs, enlarged, yellowish mottled livers and discolored hearts, were also described in experimentally infected chicks by Lewis and Hitchner (68). Goetz and Quortrup (40) and Shivaprasd *et al*. (96) have described caseous cores (Fig. 16.8) in the ceca similar to those seen in pullorum disease. Hinshaw and McNeil (51) observed that *S. enterica arizonae*-infected adult turkeys had a small amount of caseous exudate in the abdominal cavity and cystic ovules.

Histopathology

Microscopically, there is moderate to severe fibrinosuppurative inflammation associated with Gram-negative bacteria in the yolk sac and yolk stalk. In the brain, there is severe meningitis with infiltration of heterophils mixed with fibrin and bacterial colonies (Fig. 16.9). Similar exudate can also be seen in the ventricles of the brain, and there is malacia, inflammation and vascular thrombosis in the cerebral cortex. Interestingly, changes in other organs are minimal, such as necrosis of hepatocytes and infiltration with mononuclear inflammatory cells, increased numbers of mononuclear phagoctyic system cells in the spleen, and vascular congestion in various organs. Occasionally inflammation of the pericardium, air sacs, synovium, and intestine especially the ceca due to *S. arizona enterica* can be seen (96).

Otitis interna with bacteria (Fig. 16.10) and neuritis and ganglionitis of the vestibulocochlear nerve (VIIIth cranial nerve) most likely due to spread of *S. enterica arizonae* from the brain meninges in 9- to 21-day-old turkey poults have been described (97).

Pathogenesis

There is little or no information available regarding the pathogenesis of *S. enterica arizonae* but it can be assumed that it causes

disease similar to other salmonellae. Being a Gram-negative bacteria it can elaborate endotoxins causing inflammation in various organs most notably in the yolk sac, ceca, brain, eye, etc. *S. enterica arizonae* can invade the bloodstream, especially of young fowl resulting in high mortality (18).

Virulence Factors

The virulence factors of *S. enterica arizonae* are poorly understood. However recent study has shown that the *spv* virulence locus is present in *S. enterica arizonae* and that it was found to be located on the chromosome (69). Sequence analysis of the *spv* locus revealed that it contains homologues of spv^{RABC} but lacks spv^{D} . The Spv^{B} protein is an ADP ribosyltransferase that modifies actin and destabilizes the cytoskeleton of infected cells. This may explain the ability of *S. enterica arizonae* to cause disseminated infection in various species of animals including humans (69)

Diagnosis

High mortality, neurologic signs, blindness coupled with gross lesions in turkey poults can be used for a presumptive diagnosis of AA. These clinical signs, as well as lesions, however, can be seen in other *Salmonella* infections, including those of paratyphoid organisms. The organisms can usually be recovered from liver, spleen, heart blood, unabsorbed yolk sac, intestine, lung, kidney, brain, and eye.

Isolation and Identification of Causative Agent

Cultural procedures identical to those outlined and discussed under Paratyphoid Infections are employed for isolation and identification of arizonae. Standard methods for isolation and biochemical or serologic identification of *S. enterica arizonae* from poultry tissues, eggs and embryos, and environmental samples have been described (21, 26, 101, 109). Bismuth sulfite medium can be used for plating enrichment broths in addition to BG sulfa if desired. The two serotypes of *S. enterica arizonae* common in turkeys are slow lactose fermenters and, therefore, identical to paratyphoids on initial isolation on BG agar.

Selenite cysteine broth may be used in enrichment of fecal and organic tissue cultures (63, 93, 94). Selenite broth incubated at 43°C yielded fewer isolations of arizonae than did tetrathionate or selenite F broth at 35°C (44).

Forty-nine strains of *S. enterica arizonae* isolated from turkeys all had similar cultural and biochemical characteristics, varying only in use of citrate and melibiose (102). Most *S. enterica arizonae* strains were sensitive only to chloramphenicol and nalidixic acid among the antibiotics tested. Kumar *et al.* (65) found that selenite BG with sulfapyridine (SBGS) and tetrathionate BG broths gave comparable results with *S. arizonae,* but at 48 hr there was considerable reduction in recovery of arizonae from SBGS in tubes initially inoculated with high numbers of organisms. Littell (70) described a differential plating medium for isolation of *S. arizonae* that produces a uniform reaction of both lactose-negative and lactose-positive *S. arizonae*, and differentiates them from other salmonellae.

Snoeyenbos and Smyser (100) believed that litter culturing may aid epidemiologic studies and identify infected turkey flocks as part of a control program. Greenfield and Bigland (45) noted that culture of turkey litter might be a useful means of detecting insidious AA.

Culture of turkey shell membranes and shells is recommended over yolk material for rapid detection of arizonae-contaminated eggs (13, 47, 87).

Serology

Serologic analysis of cultures is essential in epizootiologic studies of AA of fowl; cultures can be submitted to the Salmonella Serotyping Laboratory, National Veterinary Services Laboratories P.O. Box 844, Ames, IA 50010, for biochemical characterization and antigenic typing.

Edwards and Galton (15) noted that it is essential to use a polyvalent *S. enterica arizonae* antiserum in preliminary examination of cultures, since arizona types may not be agglutinated by *Salmonella* polyvalent antiserum. Kowalski and Stephens (63) employed formalinized broth cultures and *S. enterica arizonae* polymonophasic antiserum in serologic identification of arizonae cultures. Snoeyenbos and Smyser (100) used *S. enterica arizonae* flagellar polyvalent, *Salmonella* flagellar Z32, and *Salmonella* somatic 18 antisera in screening cultures suspected to be *S. enterica arizonae.*

Differential Diagnosis

The clinical signs, as well as lesions of AA can be seen in other *Salmonellae* infections, including those of paratyphoid organisms. Neurologic signs can also be caused by Newcastle disease, aspergillosis, and vitamin E deficiency (encephalomalacia). Blindness in turkey poults can be due to aspergillosis or other causes such as cataracts. Therefore, AA must be confirmed by isolation and identification of the causative bacteria.

Prevention and Control

Because *S. enterica arizonae* is egg transmitted, primary breeding stock must be developed free of *S. enterica arizonae.* The control program at the multiplier breeder level is dependent on having available *S. enterica arizonae*-free stock. Management procedures outlined under Paratyphoid Infections are applicable for the control and reduction of AA. The program outlined by Ghazikhanian *et al.* (38) for the primary breeder level is applicable to the multiplier level except for the treatment of hatching eggs with antibiotics. Total confinement, bird proof and rodent proof buildings that can be cleaned and disinfected, quality feed and feed ingredients, and microbiologic monitoring at the hatchery and breeder farm levels are essential.

Ghazikhanian *et al.* (38) reviewed the program of a primary breeder to reduce and eliminate *S. enterica arizonae* from a basic breeding operation. A combination antibiotic hatching-egg treatment (dipped and injected) was successful in producing *S. arizonae*-free pedigree stock (37, 74). In addition to the egg treatment program, an autogenous oil-emulsion *S. enterica arizonae* bacterin was used on infected flocks to reduce transmission.

Because of contamination of ranges, a new capital building program was initiated (total confinement, paved floors, bird proof). A cleaning and disinfecting program was initiated after each depopulation and the facilities were monitored to determine the effectiveness of the program. Finally, special emphasis was placed on frequent egg-collection practices. Only pelleted feed containing no animal or poultry by-products was used. The program has been highly successful.

Serologic Testing

Serologic tests have not been entirely effective in detecting or controlling AA in turkeys (1, 86, 112).

Methods for preparing and using *S. enterica arizonae* antigens for serologic testing of chickens and turkeys have been outlined (4).

Timms (101) found that the most reliable and satisfactory methods for detecting *S. enterica arizonae* at various stages of infection in adult turkeys were the rapid serum plate (SP) test and the somatic tube agglutination (TA) test. The rapid whole-blood (WB) test was found to be a useful tool in testing large numbers of birds in the field, but it required confirmation by the TA test. Uses of the agar gel diffusion, indirect hemagglutination, immunofluorescene, and H agglutination tests in providing supporting evidence of infection were discussed. Lamont and Timms (67) reported use of O and H TA tests, rapid WB test, and agar gel precipitin tests for detection of AA in turkeys. They also found the rapid WB test particularly useful for flock screening.

Sato and Adler (93, 94) used a formalin-treated broth culture of actively motile arizona strains in preparing H antigen, and ethanol-treated cell suspension from beef heart infusion agar for O antigen. They found that naturally infected turkeys had positive O agglutination reactions at some time during the period they were observed; however, some of the same birds were negative when tested with H antigen. The H agglutinins disappeared earlier than O agglutinins. Not all infected birds revealed positive O agglutination tests at time of necropsy. There was little correlation between serologic results and persistence of infection.

Kumar *et al.* (66) developed a tetrazolium-stained microagglutination (MA) test antigen for detection of *S. arizonae* infections in turkeys. The MA test was demonstrated to be far more sensitive and superior to the TA and SP tests in detecting turkeys infected with *S. enterica arizonae.* Attempts to detect infection with the microantiglobulin test were unsuccessful.

Adult carriers may lack detectable antibodies 12–14 wk after exposure, and infected turkey hens go through an antibody-negative phase at 16–20 wk of age when most breeder flocks are tested (66, 101). When the ovary becomes activated following a lighting regime at 28–32 wk of age, antibodies may be detectable. At that stage in the breeding cycle, it is too late to eliminate the flocks. Greenfield (42) noted that antibody titers do not persist for lengthy periods and may not be detectable in birds with subclinical infections.

An enzyme-linked immunosorbent assay (ELISA) using outer-membrane proteins extracted from *S. enterica arizonae* as antigens was found by Nagaraja *et al.* to be sensitive and specific for the detection of *S. enterica arizonae* infection in breeder flocks of turkeys (77, 79). It was considered to be a valuable tool to determine which breeder flock is infected, allowing the hatchery program to be adjusted to reduce *S. enterica arizonae* dissemination at the time of hatching.

Vaccination

Several types of bacterins have been applied to turkey breeding stock. Holte (54) found that vaccinated breeders exposed to *S. arizonae* 18:Z4,Z32 had reduced shedding and were protected from systemic infection, thus, preventing egg transmission of arizonae. Parental immunity was found to be transmitted to poults of vaccinated hens.

Sato and Adler (92) found varying degrees of protection afforded by arizona bacterins in both mice and turkeys. A formalin-treated whole culture in aluminum hydroxide gel provided the best protection, based on the number of organisms that migrated to the spleen following intramuscular challenge. In turkeys, a chrome-alum-treated arizona bacterin provided protection against both oral and intraperitoneal challenge (76). Fecal shedding for the first 3 wk after challenge may be reduced by immunization with bacterins (1).

Gerlach *et al.* (36) found serum from nonimmunized turkey hens had both bacteriostatic and bactericidal effects on cultures of *S. arizonae* 18:Z4,Z32, but there was no inhibitory activity in the serum of birds vaccinated with arizona bacterin or in serum from naturally infected breeders. Inhibition of growth was not associated with presence of agglutinating antibodies; in fact, the opposite appeared to be true. In contrast, a bactericidal substance in the albumen of eggs from vaccinated turkeys was reported (1).

Ghazikhanian *et al.* (38) reported encouraging results using oil-emulsion bacterins; egg transmission following challenge was reduced from 12% in nonvaccinated controls to 2% in vaccinated turkeys. Vaccination against *S. arizonae* infection with a mineral oil-adjuvant vaccine was evaluated in turkey breeder flocks under laboratory and field situations by Nagaraja *et al.* (78, 80). The results were encouraging; it was possible to obtain *S. arizonae*-free progeny from vaccinated breeder flocks held in infected environments.

Lowry *et al.* (71) reported that immunoprophylactic administration of *S.* Enteritidis-immune lymphokines in turkey poults significantly reduced the horizontal transmission and organ invasion of *S. enterica arizonae.*

Treatment

Chemotherapy may reduce losses in acute outbreaks of AA and may be recommended to prevent spread of the disease in market flocks. Williams (106) reviewed various treatments for AA. In the United States, the only drugs approved by the Food and Drug Administration for treatment of AA are antibiotic injectables, gentamicin and spectinomycin. These injectables, given at the hatchery, have dramatically controlled the acute losses and morbidity that may occur during the first 3 wk of age. Isolates of *S. enterica arizonae* resistant to gentamicin have been reported (20, 53). Addition of 30 ppm of zinc from zinc-methionine to the diet of young turkeys increased the reduction of intravenously administered *S. arizonae* from the spleen (61).

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Chapter 17

Campylobacteriosis*

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Introduction

Avian species, especially domestic poultry including chickens, turkeys, ducks, and geese, are frequently infected with the members of thermophilic *Campylobacter*, primarily *Campylobacter jejuni* and *Campylobacter coli* (172, 179, 196). As enteric organisms, *C. jejuni* and *C. coli* are well adapted to the avian host and reside in the intestinal tract of birds. Despite extensive colonization, *Campylobacter* infections produce little or no clinical diseases in poultry (40, 107, 137). However, infectious hepatitis associated with *C. coli* and *C. jejuni* has been reported in ostriches, causing a high morbidity and mortality (194).

Although thermophilic campylobacters are not significant pathogens for poultry, they are of importance to food safety and public health, with *C. jejuni* being responsible for the majority of human campylobacteriosis, followed by *C. coli,* and rarely by *C. lari*. *Campylobacter* has now emerged as a leading bacterial cause of foodborne gastroenteritis in humans around the world (124). Most of *Campylobacter*-related illnesses in humans are sporadic and characterized by self-limiting watery and/or bloody diarrhea, abdominal cramp, and possible fever; however, severe conditions may occur in immunocompromised patients, requiring antibiotic treatment (61, 124). In addition, *Campylobacter* infection is associated with Guillain-Barre syndrome, a postinfectious autoimmune disease characterized by acute and progressive neuromuscular paralysis(103, 131).

The high prevalence of *Campylobacter* in the intestinal tract of market-age poultry results in frequent contamination of poultry carcasses in the processing plants. Thus, poultry products at retail are often contaminated by *Campylobacter* (40, 85, 91, 95, 223, 232). Handling and eating raw or undercooked poultry meat is considered a significant risk factor for human campylobacteriosis (8, 46, 60, 61, 120, 224). In addition, many avian *Campylobacter* isolates have become resistant to clinically important antimicrobials such as fluoroquinolones and macrolides (14, 65, 115, 230), which may potentially result in treatment failures in patients receiving antibiotic therapy. Therefore, reduction or elimination of *Campylobacter* from poultry and poultry products constitutes a major effort in improving food safety.

Etiology

At present, the genus *Campylobacter* contains 16 species, with *Campylobacter fetus* being the type species (141). Based on recent extensive DNA-rRNA hybridization studies and 16S rRNA sequence data, the family Campylobacteraceae was found to represent a diverse but phylogenetically distinct group, rRNA superfamily VI, within the group of gram-negative bacteria (141, 212). This lineage is also known as the epsilon division of the Proteobacteria, and comprises rRNA homology groups I (*Campylobacter* and *Bacteroides ureolyticus*), II (*Arcobacter*), and III (*Helicobacter* and *Wolinella succinogenes*). Members of this lineage are characterized by their low chromosomal G + C content, inability to ferment carbohydrates, and microaerobic growth requirements.

The members of the genus *Campylobacter* are associated with a wide variety of diseases in humans and animals although they are commensals in poultry (187). Within the genus, three species (*C. jejuni*, *C. coli*, and *C. lari*) known as thermophilic *Campylobacter* are of clinical significance as they are the dominant causative agents of human campylobacteriosis (61,132).

Thermophilic *Campylobacter* spp. grow optimally at 42°C on artificial media (132, 179). They are slowly-growing fastidious organisms, and require a microaerobic atmosphere for optimal growth (132, 154, 205). In general, *Campylobacter* is sensitive to oxygen, desiccation, osmotic stress, low pH, and high temperatures (59, 144). *Campylobacter* cells are S-shaped spirally curved rods in size of 0.2 to 0.8 μm wide and 0.5 to 6.0 μm long, although cells may transform to spherical or coccoid forms in response to stress or deleterious conditions (132, 179). The members of the genus are Gram-negative, nonsporeforming, and posses a single polar flagellum, mediating a characteristic corkscrew-like or darting motility (132,179). *Campylobacter* spp. are unable to ferment or oxidize carbohydrates, and thus energy is derived from the degradation of amino acids or tricarboxylic acid cycle intermediates (99).

Pathobiology and Epidemiology

Incidence and Distribution

C. jejuni and C. coli are widespread in avian hosts (40, 136, 172, 196). Generally, the carriage rate of *Campylobacter* in domestic poultry is found to be much higher than that in wild birds (179,

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196, 206, 227). This is probably due to the high bird density in commercial poultry houses, which facilitate the spread of *Campylobacter* between birds. The numbers of *Campylobacter* positive poultry flocks are generally high, but vary by regions, seasons, and the production types (conventional, free-range, and organic, etc.). It appears that the prevalence of *Campylobacter* is lower in Scandinavian countries (e.g. Norway, Sweden, Finland, and Iceland) than in other European countries, North America, and developing countries (137). Many prevalence studies have been conducted in Europe and the United States, which reported *Campylobacter*-positive flocks ranging from 3% to 97% (13, 17, 20, 26, 56, 68, 72, 74, 79, 82,97, 149, 161, 198, 209, 219, 225). Despite the fact that the majority of on-farm surveys were conducted with broiler chickens, breeder flocks and laying hens are also commonly infected by *Campylobacter* (88, 170, 179).

Seasonal variations were observed in the prevalence of *Campylobacter* flocks with a peak in warm months (17, 26, 161, 198, 219). The exact reason(s) for this seasonal variation is unknown, but it is proposed that the peaking prevalence of *Campylobacter* in warm months is due to increased fly population and fly-mediated transmission (67). Although this hypothesis is plausible, direct evidence is still needed to prove that flies are an important vector in transmitting *Campylobacter* in summer. On commercial poultry farms, *Campylobacter* is rarely detected in birds < 3 weeks of age. Typically, the prevalence of *Campylobacter* increases as the birds grow and reaches to the highest point at the slaughter age for broiler chickens. There is a general trend that *Campylobacter* is more prevalent in organic and free-range flocks than in conventional productions (53, 74, 115, 163, 211, 225). For organic and free-range operations, birds have free access to the outside environments and are slaughtered at an older age, both of which may account for the increased prevalence rates of *Campylobacter*. Once a broiler flock is infected with *Campylobacter*, the majority of the birds within that flock can become colonized in a short time period (17, 20, 32, 64, 198).

With respect to species distribution of campylobacters isolated from chickens, *C. jejuni* accounts for the majority of isolates, followed by *C. coli*, and rarely by *C. lari* (20, 26, 28, 32,56, 74, 82, 115, 161, 164, 179, 185). However, a higher or even sometimes predominant proportion of *Campylobacter* isolates from turkeys or organic and free-range productions are *C. coli* (74, 115, 164, 225). The isolation of other *Campylobacter* spp. including *C. upsaliensis* and *C. hyointestinalis* in poultry is at a low rate (179, 219). Poultry *Campylobacter* isolates are of multiple genotypes and great genetic diversities. Chicken flocks can be colonized by a single or multiple genotypes of *Campylobacter* (20, 28, 54, 77, 137, 145, 164, 199, 225). Even during a single rearing cycle, a broiler flock can be infected by different species or genotypes of *Campylobacter* at different time points (28, 72, 164, 172), reflecting the dynamic changes of *Campylobacter* populations on poultry farms.

Transmission, Carriers, Vectors

Horizontal Transmission

Many farm-based studies have suggested that horizontal transmission from the environment to poultry houses is the most common mode of transmission of *Campylobacter* on poultry farms. Potential sources of infection include old litter, untreated drinking water, other farm animals, domestic pets, wildlife species, house flies, insects, equipment and transport vehicles, and farm workers. Since *Campylobacter* growth is very sensitive to oxygen and temperature, the organism is usually unable to grow in feed, litter or water under normal ambient conditions (82, 85, 98). The organism is usually absent in fresh litter or feed before broilers are infected. Used litter may become contaminated by *C. jejuni* and may play a role in maintaining *C. jejuni* in the farm environment (112, 127). However, surveys conducted in the USA and Europe indicated that there were no marked differences in the prevalence and onset time of *Campylobacter* shedding among flocks on different farms with different litter usage practices (55, 198). These findings suggest that used litter may play a role in transmitting *Campylobacter*, but is not the only source of *Campylobacter* for commercial poultry production. Due to its low moisture content, feed is unlikely an original source for the introduction of *C. jejuni* into the poultry houses (90, 210). However, feed can be contaminated by feces in chicken houses (64), which may facilitate the spread of *Campylobacter* within production facilities.

Unchlorinated water supplies have been implicated as a source of *Campylobacter* infection in broiler chickens (97, 146). Due to its requirement for microaerobic conditions and its inability to grow below 31–32°C (69), *C. jejuni* is unlikely to propagate in environmental water. The presence of this organism in water systems is probably a sign of a recent contamination by feces of livestock or wild birds (93, 191). Therefore, it is likely that contaminated water serves as a passive carrier of *Campylobacter* rather than a niche for growth of *Campylobacter*. Also, drinking water on poultry farms usually becomes positive with *C. jejuni* only after chickens are colonized (20, 210, 233), questioning the role of drinking water in transmitting *Campylobacter* on poultry farms. Water supplies of intensively reared broilers are often inhabited by protozoa. It was shown that *Campylobacter* could enter into protozoan cells and was able to survive for prolonged periods inside the protozoan cells (15, 190). The same studies also found that *Campylobacter* was more resistant to disinfectants (e.g., chlorine) when co-cultured with protozoa than when cultured alone. These results suggest that protozoa in the aquatic environments could serve as a potential reservoir for *Campylobacter* and may facilitate the survival and transmission of *Campylobacter* in animal reservoirs.

Insects (houseflies, darkling beetles, cockroaches, mealworms, etc) can act as mechanical vectors, and may transmit *Campylobacter* to poultry houses (90, 161, 165). Several studies reported that identical serotypes and genotypes of *Campylobacter* were isolated from both broiler and insects within broiler houses (19, 90, 166). There were also reports indicating that insects in poultry houses were not positive for *C. jejuni* until the organism was isolated from broilers chickens in the same houses (19, 135). A recent study by Hald *et al.* (67) reported that in summer months about 10% of flies around a broiler house were contaminated with *Campylobacter* and that hundreds of flies could enter a poultry house through the ventilation system. Further-

more, the same study demonstrated that *Campylobacter* isolates from the broiler chickens and from flies captured in the environment were of the same genotypes. These findings suggest the possibility that flies serve as a vector for transmitting *Campylobacter* on farms, especially during the summer months.

Several studies have shown that rodents and other small wild animals such as raccoons harbor *Campylobacter* in their intestine, and thus these wild animals can potentially introduce *Campylobacter* into grow-out houses (12, 97, 135). In the study reported by Petersen and Wedderkopp (151), the persistence of some *C. jejuni* clones during successive broiler flock rotations was suggested to be a result of survival of the organism in such reservoirs as rodents and insects that were able to evacuate the house during cleaning and disinfection and then return. However, no direct evidence existed to prove this theory (64, 90, 210). In addition, other studies found that *Campylobacter* was not isolated from rodents in the vicinity of broiler houses (64,92). Considering the fact that vermin control programs are implemented in most commercial poultry production facilities, rodents/small animals are unlikely a common source of *Campylobacter* infection for broiler flocks.

Campylobacter has a wide distribution in wild birds (109, 160, 215). Wild birds in the vicinity of poultry production facilities are often found to be infected with *C. jejuni*; however, the *Campylobacter* isolates from wild birds are usually different from those of chicken origin (64, 135, 166). Since wild birds often carry *Campylobacter* in their intestines, and owing to their great mobility, wild birds may spread *Campylobacter* to domestic poultry through fecal contamination of pastures, forage, surface water, or feed.

Presence of other farm animals on broiler farms including pigs, cattle, sheep, and fowl other than chickens has been found to be associated with an increased risk of *Campylobacter* infection in broiler chickens (20, 26, 97, 210). In the study by Gregory *et al.*, cattle and chickens on the surveyed farms were found to be concurrently infected with *C. jejuni* (64). In a follow up study, *C. jejuni* isolates from these cattle were shown to have the same *flaA* type as the isolates from the broilers on the same farm (199). Identical genotypes between cattle and broiler isolates from the same farm were observed in another study, and cattle were suggested to be a source of infection to the broilers on the farm (210). However, as it was indicated by the authors (210), the direction of the transmission was unknown, and the vectors/carriers mediating the transmission between cattle and chickens were not revealed. In other studies, *C. jejuni* isolated from cattle was found to be different from the isolates recovered from the broilers on the same farm (28, 87, 90, 135, 166), suggesting that cattle and broilers were infected by *Campylobacter* from different sources. Pigs are also frequently colonized by *Campylobacter* (12, 64, 135). Tending pigs before entering broiler houses was indicated as a risk factor for *Campylobacter* colonization of chickens (97). Some earlier studies found pigs and broilers to be infected with the same serotype of *C. jejuni* (12, 166), while studies using more discriminatory typing tools showed that pigs and broilers on the same farm were usually infected with different strains of *C. jejuni* (89, 90, 199, 208, 210). Also, pigs are usually infected with *C. coli* instead of *C. jejuni* (22, 204), while poultry (especially chickens) are frequently colonized by *C. jejuni* (196, 210). Other farm animals (e.g. sheep and horses) and pets (e.g. cats and dogs) can also be infected with *C. jejuni* (196); however, their potential role as a source of broiler infection has not been established.

Farm workers may carry *Campylobacter* into poultry houses (20). *Campylobacter* was isolated from footbath water, farmer's boots, and transport crates (28, 198, 210). Therefore, it is possible that *Campylobacter* may spread between broiler flocks or farms by the movement of personnel or farm equipment.

In summary, it appears that poultry houses can be invaded by *Campylobacter* in many different ways from various sources. The complexity of *Campylobacter* transmission and the widespread presence of *Campylobacter* in the production system greatly undermine the success in control of *Campylobacter* on poultry farms by using management-based strategies.

Vertical Transmission

It has been a major debate if vertical transmission plays a role in introduction of *Campylobacter* to poultry flocks. The current notion is that vertical transmission of *Campylobacter* does not occur or occurs very rarely. Underlying this argument are several reasons. First, young broiler chickens usually lack *Campylobacter* before 2 or 3 weeks of age even though they are hatched from eggs originated from breeder flocks infected by *Campylobacter* (17, 19, 28, 182, 208, 210). Second, progeny broiler flocks are frequently infected with strains different from those of their breeder flocks (28, 34, 150, 210). Third, chicken flocks originating from the same parent flocks do not always show similar serotypes (20), but broilers from different hatcheries may be infected with the same *Campylobacter* clones (151). A well-conceived longitudinal study conducted in Iceland by Barrios reported the lack of vertical transmission of *Campylobacter* in poultry production (17). Finally, isolation of *Campylobacter* from eggs has been scarce, and to date no studies have reported isolation of live *Campylobacter* cells from hatcheries or young hatchlings (48, 76, 84, 170, 182).

However, there are some studies suggesting that vertical transmission of *Campylobacter* may occur from breeder flocks to progeny broilers. Some earlier studies showed that *C. jejuni* could be isolated from both the outer (48) and inner (182) surface of eggshells laid by naturally infected commercial layers or broiler breeders. Shane *et al.* (180) isolated the organism from both the interior surface of eggshell and egg contents after swabbing feces containing *C. jejuni* onto the surface of the eggs. Following experimental infections of eggs with *C. jejuni* by either the temperature differential method (37) or inoculation of egg albumen via direct injection (182), the organism was recovered from both the contents of unhatched eggs and from the newly hatched chicks. Investigations using sensitive molecular detection methods demonstrated the presence of *Campylobacter* DNA in embryos and newly hatched chicks (35, 36, 84), and in hatcheries (76). Furthermore, *C. jejuni* has been isolated from the reproductive tract of healthy hens (27, 31, 75, 87) and from semen of commercial broiler breeder roosters (43). The same

genotypes of *C. jejuni* have been identified from breeder flocks and their progeny flocks (28, 42, 84, 147). These studies suggested the possibility of vertical transmission, but did not prove that vertical transmission actually happened.

Incubation Period

Birds can be readily infected by *Campylobacter* naturally or experimentally; however, the infection usually does not cause clinical diseases, and C*ampylobacter*-associated diarrhea in poultry is a rare event. Experimental studies demonstrated that colonization could occur as early as one day after inoculation (18, 102, 171, 183, 197, 220, 229). In a few cases where diarrhea was observed, the incubation time ranged between 2 to 5 days (167, 175, 220). The minimal infective dose to establish colonization in day-old chicks was shown to be as low as 2 cfu (102), although other studies indicated higher infectious doses (183, 197, 229). Once *Campylobacter* colonization is established, it can persist in the intestinal tract for multiple weeks (5, 102, 172), but gradual decrease in the level of colonization usually occurs after a prolonged plateau period (116, 171, 196).

On poultry farms, *Campylobacter* is rarely detected in birds of less than 2–3 weeks of age. The reason for this lack of infection in young birds is unclear and may be related to multiple factors including the presence of maternally-derived antibodies (171, 173) or differences in environmental or host-related factors. Once a flock is infected, *Campylobacter* spreads rapidly within the flock, leading to colonization of the majority of the birds within a few days (20, 56, 64, 184). Despite the fact that *Campylobacter* infection rarely occurs in young flocks on poultry farms, newly-hatched chickens can be readily infected experimentally with *Campylobacter* (172, 179, 183).

Clinical Signs and Pathological Lesions

Campylobacter infections in poultry usually produce no clinical signs of disease under natural conditions. However, it has been reported that in ostriches natural *Campylobacter* infection can cause clinical illness and pathological lesions in liver and intestines (136, 194). Vibrionic hepatitis was prevalent during 1950s and 1960s in commercial laying hens but is only occasionally reported nowadays (29, 44). It was suspected that *Campylobacter* might be the cause of the disease, but the etiologic agent(s) for vibrionic hepatitis was not formally identified (181).

Some studies reported that experimental challenge of young chickens with *Campylobacter* can induce clinical diseases including watery/mucoid/bloody diarrhea, weight loss, or even mortality (167, 175, 220). In an early report, 3-day-old chickens inoculated with a high dose of *C. jejuni* developed diarrhea within 72 h, which lasted for 10 days and resulted in considerable weight loss as well as a mortality of 32% (167). Welkos (220) reported that almost one-third of the day-old chicks and nearly all of the newly-hatched chicks, but none of the 3-day old chickens, developed signs of gastroenteritis after challenge orally with *C. jejuni*. Similarly Sanyal *et. al*. (175) observed watery/mucoid diarrhea in 81% of 36 to 72-hr-old birds 5 days after inoculation with *C. jejuni*, and also found that the Starbro strain of chickens was more likely to develop diarrhea than the white leghorns strain. *Campylobacter* infection in commercial broilers of less than 2 weeks of age (a rare event) was found to be associated with diarrhea, decreased weight gain, and excess mortality (134). Another study using newly hatched or 4-day-old turkey poults also observed reduced weight gain and transient watery diarrhea in the birds after inoculation with *Campylobacter* (106). Oral inoculation of 3-week-old Japanese quails with *C. jejuni* resulted in diarrhea that lasted for 2 weeks (119). Despite these isolated reports, many other studies did not observe any clinical diseases associated with *Campylobacter* infections in poultry (18, 102, 171, 183, 197).

Gross pathologic lesions associated with *Campylobacter* infection in experimentally infected chicks are minimal and mainly confined to the gastrointestinal tract. Due to accumulation of fluid, gas, or excess mucus, distention of intestines including ceca with watery/foamy material may be a common finding (175, 220). Blood and mucus in the lumen of small intestine, and petechial hemorrhages in the gizzard mucosa of chicks can be seen occasionally (220). There is a report that *Campylobacter* was isolated more frequently (21% of 223 livers) from broiler chicken livers with necrotic lesions than from normal livers (12% of 50 livers) obtained from slaughter plants in Canada (25); however, there was no evidence that *Campylobacter* directly contributed to the lesion.

Microscopic lesions following experimental infection of chicks are mostly unapparent or minimal, but exceptions occur in birds with severe clinical and gross pathological signs. Usually examination of gastrointestinal tissue reveals no necrosis or invasion of the epithelium or any other pathological changes; however, a mild edema of the lamina propria and submucosa of the intestines, mostly in ceca, was reported with *Campylobacter* infections in chickens (18, 102, 175, 183, 197). In some cases, *Campylobacter* cells can be seen attaching to the brush borders on enterocytes, within intestinal epithelial cells, and inside or outside of the cells of lamina propria with minimal tissue or cell damage (167, 175, 220). Mononuclear infiltration in the submucosa and villous atrophy resulting in accumulation of red blood cells and leucocytes in small and large intestinal lumen may occur in more severe cases (220).

Pathogenesis of the Infectious Process

Birds become infected with campylobacters via the fecal-oral route. As enteric organisms, *Campylobacter* spp. are able to survive the harsh conditions in the stomach (gizzard) as well as in small intestine and eventually reach the lower intestines, where the organisms colonize in cecal and cloacal crypts (4, 18, 172). To a lesser extent, the organism can also be recovered from the small intestines and the gizzard, and infrequently from the liver, spleen, blood, and gallbladder (4, 96, 175). Several distinct features are associated with colonization of *Campylobacter* in chickens. First, it appears that *Campylobacter* does not adhere directly to intestinal epithelial cells, but mainly locates in the mucous layer of the crypts (18, 126). Second, usually no gross or microscopic lesions are induced in the chickens. Third, invasion of the intestinal epithelium rarely occurs with *Campylobacter.* Even when the invasion of internal organs occurs in some cases, no

clinical signs of illness are observed (18, 102, 229). Once a broiler chicken becomes infected, large numbers of the organism (up to 10^9 cfu/ g feces) can be detected in ceca and excreted in feces for a prolonged period (172).

It is likely that many genetic factors contribute to the colonization of *Campylobacter* in poultry. Published studies using genetically defined mutants revealed that flagella, DnaJ (heat shock protein), CiaB (*Campylobacter* invasin antigen B), PldA (phospholipase A), CadF (*Campylobacter* adhesin to fibronectin), CmeABC (multidrug efflux pump), MCP (a methyl-accepting chemotaxis protein), RpoN (sigma factor), the Kps locus (capsule biosynthesis proteins), the Pgl locus (protein glycosylation system), SOD (superoxide dismutase), Fur (ferric uptake regulator), and CbrR (a response regulator) all contributed to *Campylobacter* colonization in chickens (63, 70, 104, 111, 133, 158, 218, 234, 235). *C. jejuni* produces a cytolethal toxin (CTD), which is suggested to be a potential virulence factor of *Campylobacter* (16, 57, 216). Although most *Campylobacter* isolates from poultry harbor the *cdt* genes and produce toxic activity *in vitro* (16, 57), the role of CDT in colonization of chickens has not been established.

Immunity

Despite the commensal relationship between *Campylobacter* and the avian host, the infection indeed elicits both systemic and mucosal humoral responses (33, 130, 138, 162, 221). Following experimental infection of day-old chickens via oral gavage, production of *Campylobacter*-specific IgM and IgA antibodies in serum reached significant levels within 1–2 weeks of infection and peaked at weeks 4–6 postinfection, followed by gradual decreases as birds age (33, 130). In contrast, detectable levels of IgG responses developed later than IgM and IgA responses, peaked at 8–9 weeks of the infection, and persisted for a longer period (33, 130). Naturally occurring *Campylobacter* colonization in chickens also elicits overt immune responses, and anti-*Campylobacter* antibodies readily transfer from hens to their progenies as maternally-derived (173, 228). Maternal antibody plays a partial role in protecting young chickens from infection by *Campylobacter* (171). A wide variety of *Campylobacter* antigens are recognized by chicken sera (33, 162, 173, 222). There is a trend that with the development of specific anti-*Campylobacter* antibodies, the level of *Campylobacter* colonization diminishes and some infected chickens eventually clear the infection (4, 96, 137, 171, 197). However, the nature of protective immunity has not been elucidated, and it is unknown if humoral immunity or cellular immunity (or both) contributes to the clearance of *Campylobacter* from the host. To date there are no reports documenting cellular immune responses induced by *Campylobacter* infection in poultry.

Diagnosis

Culture-based Isolation and Detection Methods

Thermophilic campylobacters are fastidious and slow-growing, requiring microaerobic atmosphere (containing 5% O_2, 10% CO_2, 85% N_2) and elevated temperature (42°C) for optimal growth under laboratory conditions (41, 50, 174, 186). Thus, culturing *Campylobacter* spp. from fecal or environmental materials with a high level of background flora requires the use of selective culture media and special culture conditions. The first selective medium for culturing *C. jejuni* and *C. coli* was developed in 1977 by Skirrow (186). Since then, approximately 40 solid and liquid selective media for culturing of *Campylobacter* from clinical and food samples have been reported, which have been reviewed by Corry *et al*.(41). Some of the most commonly used ones are Skirrow, Preston, Karmali, modified charcoal cefoperazone deoxycholate agar (mCCDA), cefoperazone amphotericin teicoplanin (CAT) agar, Campy-CVA (cefoperazone vancomycin amphotericin), and Campy-Cefex medium. The selective media contain a variety of different combinations of antibiotics to which thermophilic campylobacters are intrinsically resistant, such as polymyxin, vancomycin, trimethoprim, rifampicin, cefoperazone, cephalothin, colistin, cycloheximide and nystatin. The multidrug efflux pump CmeABC in *Campylobacter* contributes, at least partly, to the intrinsic resistance to these selective agents (110). Use of these antibiotics inhibits the growth of many background microbial flora present in samples and allows the isolation of slow-growing *Campylobacter* spp.

Since *Campylobacter* spp. are sensitive to oxygen levels above 5%, *Campylobacter* selective media often contain various oxygen-quenching agents in order to neutralize the toxic effect of oxygen radicals (41). The commonly used oxygen quenching agents include blood (e.g. Skirrow and Campy-CVA media), a combination of ferrous sulfate, sodium metabisulfite and sodium pyruvate (e.g., Campy-Cefex agar), charcoal (e.g., mCCDA agar), and hematin (e.g., in Karmali agar).

Depending on the type of specimen, selective media can be used either for direct plating or for an enrichment step followed by plating for isolation of *Campylobacter*. An enrichment step in liquid medium followed by plating on solid agar plates is usually superior to direct plating alone for isolation of *Campylobacter* from processed foods in which bacteria are usually in relatively low numbers and/or in an "injured" state (41, 85). However, the enrichment step may not always perform better than direct plating when culturing fecal samples. Although enrichment can be used for fecal samples of cattle for better recovery of *Campylobacter* (24, 94, 192), it may greatly reduce the recovery rate of *Campylobacter* spp. from different sites of the intestinal tract of poultry (129). Musgrove *et al.* (129) compared enrichment and direct plating for isolation of *Campylobacter* from ceca and crops, which showed that direct plating of cecal samples on selective media resulted in a significantly higher recovery rate than the enrichment method. However, enrichment was slightly better than direct plating for the recovery of *Campylobacter* spp. from crop samples. When an enrichment step is used, it should be controlled for less than 24 hrs since a prolonged incubation in enrichment broth may actually decrease the isolation rate.

To isolate thermophilic campylobacters from environmental water, two methods can be used to increase the detection sensitivity. A large volume of water can be filtered through a single membrane with a pore size of 0.2 μm. Subsequently the mem-

brane can either be placed directly on a selective agar plate, or first cultured in an enrichment broth followed by selective plating (142, 146). If large particles are present in water, prefiltering with a membrane of a larger pore size may be required prior to the final filtering with a membrane of 0.2 μm. Alternatively, water samples can be concentrated by high-speed centrifugation from which the supernatant is discarded and the pellet is cultured by direct plating or enriched in broth followed by plating.

Usually typical *Campylobacter* colonies are visible on solid media after 48 h incubation (24, 41), but it may take up to 72–96 hrs to observe some slow-growing strains (41, 132, 174). Depending on the media used, colonies of *Campylobacter* spp. may appear differently. If the agar is moist, the colonies may appear grey, flat, irregular, and thinly spreading. Round, convex, or glistening colonies may be formed when plates are dry (41). Presumptive identification of thermophilic *Campylobacter* spp. can be done according to colony morphology, typical cellular shapes (spiral or curved rods), and characteristic rapid darting motility as observed under phase-contrast microscopy. The most commonly used phenotypic tests for identification of *Campylobacter* to genus or species level include biochemical tests (catalase, oxidase, nitrate reduction, hippurate hydrolysis, indoxyl acetate hydrolysis), antibiotic susceptibility patterns (nalidixic acid, cephalothin), and growth characteristics at different temperatures (25°C, 37°C, and 42°C) (140, 193). Differentiation between *C. jejuni* (hippurate-positive) and *C. coli* can be done with the hippurate test. However, hippurate-negative *C. jejuni* isolates have been reported (193), emphasizing the need for further testing of hippurate-negative strains with other methods when species identification is considered important.

Immunology-based Diagnostic Methods

Enzyme immunoassays (EIA), based on antigen-antibody interaction, have been developed for direct detection of *Campylobacter* spp. in animal feces or processed food. These EIA assays are commercially available in a very similar format to sandwich-ELISA assays, which use two different antibodies, to detect *Campylobacter* spp. directly in crude samples (78) or after a selective enrichment step (108, 174). Some examples of these commercial kits include VIDAS Campylobacter (bioMerieux), EIA-Foss Campylobacter (Foss Electric), and ProSpecT (Alexon-Trend). EIAs are not as sensitive as culture methods for detecting *Campylobacter* spp., and are not suitable for testing samples in which *Campylobacter* spp. are suspected to be in low numbers. They are more rapid than traditional culture methods and can be automated for easy handling (52, 78, 80, 157).

Nucleic Acid-Based Diagnostic Methods

DNA-based methods have been widely used for detection and identification of *Campylobacter* spp. The majority of the methods are designed in the formats of PCR tests, which can be used for culture confirmation or for direct detection of *Campylobacter* from environmental or clinical samples. Depending on the purposes, PCR primers can be designed from either variable or conserved gene sequences of *Campylobacter* spp. PCR primers directed to conserved sequences are usually used for general detection, while primers designed from variable sequences can be used for differentiation of species or strains. A variety of PCR assays targeting genus- or species-specific sequences have been developed to detect and identify *Campylobacter* spp. from poultry feces, and environmental samples (36, 73, 159, 174, 202, 213). When applied to crude samples, PCR-based tests tend to have a low detection sensitivity due to presence of PCR inhibitors in feces and food matrices and are unable to differentiate dead bacteria from live cells. However, PCR assays can be used in conjunction with conventional culture methods to improve the speed and accuracy of *Campylobacter* detection and identification.

In addition to PCR, many other molecular typing tools have been developed for epidemiological studies of *Campylobacter* in animal reservoirs. The majority of these molecular methods are used for typing or differentiation of pure *Campylobacter* cultures and are not suitable for detection purposes. Examples of commonly used molecular typing tools include pulsed-field gel electrophoresis (PFGE), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), ribotyping, and sequence-based methods [reviewed in reference (217)]. Sequence-based typing methods target the variable region of the *fla* gene (encoding the flagellin subunit), several housekeeping genes (multilocus sequence typing, MLSP), or the *cmp* gene (encoding the major outer membrane protein) (81, 118, 125, 231). Comprehensive reviews on various typing tools have been published previously (101, 217).

Public Health Significance

Campylobacter enteritis, caused primarily by *C. jejuni*, has become a leading bacterial foodborne illness in humans in the United States and other developed countries (2, 8). In the United States the incidence of laboratory-confirmed cases, as determined by the Foodborne Diseases Active Surveillance Network (FoodNet), was reported to be 12.72 cases per 100,000 in 2005 (1). The estimated annual cases of human campylobacteriosis are 2.1 to 2.4 millions in the United States (8, 61). *C. jejuni* is also frequently associated with diarrhea in patients <6 months of age in developing countries (21, 39). The rates of *Campylobacter* isolation ranged between 5 to 20% in children with diarrhea in some regions of Asia, Africa, and South America (139). In some developing countries, *Campylobacter* can be isolated as often as rotaviruses and enterotoxigenic *Escherichia coli* (51, 139, 203).

Clinical cases of human campylobacteriosis are characterized by a self-limited watery and/or bloody diarrhea; however, serious complications such as reactive arthritis (Reiter's syndrome), Guillain-Barre syndrome, osteomyelitis, nephritis, myocarditis, cystitis, pancreatitis, septic abortion, and bacteremia may occur infrequently (8, 21, 188). Deaths due to *Campylobacter* infection are rare but occur primarily in immunocompromised patients, infants, and the elderly (124).

Campylobacter-contaminated poultry meat is considered a major source of sporadic cases of human campylobacteriosis (8, 60, 61, 224). Worldwide up to 70% of human *Campylobacter* infections have been epidemiologically linked to consumption of chickens (7). Other risk factors including contact with house pets

and consumption of raw milk, untreated water, and undercooked beef or pork have been reported (21, 40). Outbreaks due to *Campylobacter* are rare and mostly associated with consumption of raw milk and contaminated surface water (38, 62, 105, 120, 152, 176).

Although *Campylobacter* is normally susceptible to various antimicrobials, increasing resistance to several antibiotics including fluoroquinolones (FQ), erythromycin, and tetracycline has been documented with *Campylobacter* isolates derived from animals and humans (14, 47, 148, 155, 168). Of particular concern is the resistance to FQ antimicrobials (65, 230). Both laboratory treatments (86, 117, 121) and farm studies (83) showed that treatment of *Campylobacter*-infected chickens with fluoroquinolones resulted in selection of FQ-resistant *Campylobacter* mutants that propagated rapidly and persisted in chickens. Many epidemiological surveys reported the prevalence of FQ-resistant *Campylobacter* in poultry in different regions of the world (45, 115, 143, 156, 230). There is a temporal link between the approval of FQ antimicrobials for animal production and the subsequent increase of FQ-resistant *Campylobacter* isolated from both animals and humans (3, 10, 153, 169, 189, 230). Resistance to erythromycin, a clinically important drug, has also been increasingly reported, especially among *C. coli* isolates (14, 47, 115, 169). As poultry is considered a main source of human *Campylobacter* infections, development of FQ-resistant *Campylobacter* in poultry is regarded as a threat to public health (11, 155, 189, 230), which has led to the recent withdrawal of fluoroquinolone use in poultry in the U.S.

Intervention Strategies

At present no effective control measures are available for prevention and control of *Campylobacter* infections on poultry farms. Several approaches including stringent biosecurity, competitive exclusion, phage treatment, bacteriocin-based treatment, and vaccination have been reported in published studies.

Biosecurity

Several epidemiological investigations have found a correlation between decreased *Campylobacter* infection in broiler flocks and the employment of stringent biosecurity measures and hygienic practices on farms (20, 32, 56, 82, 97, 164, 210). In most of these studies, adherence to biosecurity measures either reduced the colonization level or delayed the onset time of colonization of birds by *Campylobacter*, but was largely unsuccessful in preventing introduction of *Campylobacter* into broiler flocks (20, 82, 136, 164, 184). Although on-farm biosecurity measures appear to be effective in reducing the incidence of *Campylobacter* infection in North Europe such as Norway, Sweden, and Finland, these measures have met limited success in other countries such as the UK, the Netherlands, and Denmark (2, 209, 219). Because *Campylobacter* spp. are commonly present in the poultry farm environment and poultry flocks can be infected by multiple sources, it is rather difficult to eliminate *Campylobacter* from poultry houses by use of biosecurity practices alone. In addition, stringent biosecurity measures are cost-prohibitive and hard to maintain.

Competitive Exclusion

A number of studies investigated the feasibility of competitive exclusion as a mean of preventing *Campylobacter* colonization in broiler chickens (122). These studies used fecal or cecal mucus suspensions, intestinal homogenates, undefined cecal mucus culture, or defined flora from cecal mucus. Although some level of protection was observed in chickens challenged under laboratory conditions, the degree of reduction was inadequate for practical purposes (122). Ideally, competitive exclusion should use pure (defined) cultures instead of crude intestinal mucous suspensions. However, the effect of defined competitive exclusion flora on *Campylobacter* was variable and inconsistent (123, 177, 178, 195). Also, treatment of broiler chickens with pure cultures of *Saccharomyces boulardii* (113) or *Lactobacillus acidophilus* and *Streptococcus faecium* (128) was only partially successful in reducing *Campylobacter* colonization. Currently none of the commercially available competitive exclusion products appears effective for excluding *Campylobacter* from chickens under production conditions (122). In several studies assessing the effectiveness of the competitive exclusion product Broilact®, substantial reduction in *Campylobacte*r colonization was observed in one study, but was not reproduced by others (6, 66, 122).

Vaccination

Currently there are no commercially available vaccines for control of *Campylobacter* in poultry. The commensal nature of *Campylobacter* colonization and the great genetic/antigenic diversity among different *Campylobacter* strains create a great challenge for developing effective vaccines that can confer a broad-spectrum protection. The reported immunization studies used killed whole cells, flagellin-based subunit vaccines, or genetically engineered live vectors expressing *Campylobacter*-specific antigens. Most of the studies showed some, but not a biologically significant protective effect in chickens (100, 136, 162, 221). However, a recent study by Wyszynska *et al.* reported a drastic reduction of *Campylobacter* colonization by immunizing chickens with an attenuated *Salmonella* strain expressing the *C. jejuni* CjaA antigen (the substrate-binding component of a ABC transport system) (226). In this work, chickens were orally immunized with the recombinant *Salmonella*-CjaA vaccine at 1 and 14 days of age and challenged at 4 weeks with a wild-type *C. jejuni* strain. Following the challenge, most of the immunized birds had undetectable ($< 1\times10^3$ cfu/g feces) levels of *Campylobacter* organism in the ceca, while all of the nonvaccinated control birds were heavily colonized by *Campylobacter* (up to 10^9 CFU/g cecal content). It is unknown if this vaccine can provide protection against different *Campylobacter* strains, but the encouraging results suggest the possibility of using vaccination to control *Campylobacter* infection on poultry farms.

Other Intervention Strategies

Several other potential intervention strategies have been evaluated to eliminate *Campylobacter* colonization in chickens, including phage therapy, bacteriocin-based treatment, and feed/water additives (49, 114, 200, 201, 214). *Campylobacter*-specific bacteriophages are commonly recovered from broiler chickens

and the farm environment. Experimental challenge studies, in which broiler chickens were used to assess the prophylactic or therapeutic effect of bacteriophages against *Campylobacter* colonization, showed a significant decrease in *Campylobacter* numbers in chickens treated with phages (114, 214). However, the level of reduction was variable and was affected by the phage types and doses. Additional studies are needed to determine if phages can be used as an effective and practical means to control *Campylobacter* in poultry. Different diet formulations were evaluated for their effects on *Campylobacter* colonization in broiler chickens (58, 207). Although some differences were observed among the formulations, the effects were biologically insignificant. Similarly, fermented or acidified feed (71) or lactic acid treated drinking water (30) did not result in significant reduction in the level and rate of *Campylobacter* colonization in chickens. Supplementation of feed with prebiotics (e.g., lactose, fructooligosaccharide, mannose-oligosaccharide), immune response stimulators (selenium, beta-glucan), antimicrobials (flavophospholipol, salinomycin), other compounds (chlorate or nitro-based substances), or activated charcoal also had limited success in reducing the incidence of *Campylobacter* colonization in chickens (9, 23, 49). Recently Stern *et al.* evaluated the effect of bacteriocins purified from *Paenibacillus polymyxa* and *Lactobacillus salivarius* on *Campylobacter* colonization in chickens (200, 201). When given to chickens as feed supplements, both bacteriocins were highly effective in reducing *C. jejuni* infections. Notably, the bacteriocins had broad anti-*Campylobacter* activities and were effective against different strains of *C. jejuni* when tested in chickens. As the authors indicated, treatment of broiler chickens with bacteriocins before slaughter may be used to reduce carcass contamination in processing plants (200, 201).

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Chapter 18

Colibacillosis

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Introduction

Definition and Synonyms

Colibacillosis refers to any localized or systemic infection caused entirely or partly by avian pathogenic *Escherichia coli* (APEC), including colisepticemia, coligranuloma (Hjarre's disease), air sac disease (chronic respiratory disease, CRD), swollen-head syndrome, venereal colibacillosis, and coliform cellulitis (inflammatory process), peritonitis, salpingitis, orchitis, osteomyelitis/synovitis (turkey osteomyelitis complex), panophthalmitis, omphalitis/yolk sac infection, and enteritis. Lesions alone should not be used to infer an *E. coli* infection without the descriptor "coli" or "coliform" being added, because other opportunistic bacteria can behave similarly to *E. coli* in secondary infections. Colibacillosis in mammals is most often a primary enteric or urinary tract disease, whereas colibacillosis in poultry is typically a localized or systemic disease occurring secondarily when host defenses have been impaired or overwhelmed by virulent *E. coli* strains (29). Strains of *E. coli* that cause disease outside the intestinal tract of any species share common characteristics and are called extraintestinal pathogenic *E. coli* (ExPEC)(249, 449). Most APEC are ExPEC and share characteristics with mammalian ExPEC.

Several reviews on APEC and colibacillosis in poultry have been published (28, 109, 131, 186, 292, 436, 524). Earlier information on colibacillosis can be found in previous editions of *Diseases of Poultry*.

Economic Significance

There is general agreement that colibacillosis is the most common infectious bacterial disease of poultry and that collectively, *E. coli* infections in their various forms are responsible for significant economic losses. Often colibacillosis is among the most frequently reported diseases in surveys of poultry health or condemnations at processing. For example, 43% of broiler carcasses condemned for disease at processing had lesions consistent with colisepticemia (556), and colibacillosis was the major cause of infections causing condemnation of processed chickens in Switzerland (241). Flocks with airsacculitis at processing had, in general, lower average body weights of 84 g/bird, and more processing errors, fecal contamination, and *Campylobacter* contamination (447). *E. coli* was isolated from 88.2% of chickens with airsacculitis from flocks in Jordan (126). In Greece colibacillosis was the most frequent respiratory disease of broilers over a 10-year period (1992–2001) and caused considerable economic losses (155). APEC infections were identified as a major factor in poultry disease in Belgium. Data collected at the East-Flanders regional laboratory between 1997–2000 on groups of healthy and sick broilers, layers, and breeders showed the incidence of APEC infection to be 17.7%, 38.6%, and 26.9% respectively. Resistance to antibiotics was also found to be high (525). Colibacillosis was second only to enteritis as a disease of turkey flocks in California affecting 6 of 26 flocks (77). However, in spite of its recognized importance no studies to accurately define the economic significance of colibacillosis in poultry appear to have been done. In people the impact of ExPEC infections amounts to losses of, ". . . billions of health care dollars, millions of work days, and thousands of deaths annually" (450).

Public Health Significance

Most APEC isolated from poultry are specific clonal types that are pathogenic only for birds and represent a low risk of disease for people or other animals (67, 439). However, chickens are readily infected experimentally with *E. coli* O157:H7, an important enterohemorrhagic pathogen of humans that produces Shiga toxin, and can shed the organism for months (40). Infection with the organism in chickens is strain dependent and requires flagella but not intimin, the surface adhesin responsible for attachment of the organism to epithelial cells in mammals (44, 295). Natural infection with O157:H7 has been identified in both chickens and turkeys in different geographic areas (191, 212, 417). Contamination of poultry meat with this organism can occur as evidenced by a food-borne outbreak of diarrheal disease that was associated with contaminated turkey meat (40, 115, 176, 488). Free-living waterfowl can serve as carriers and be a source of *E. coli* O157. Infection of cattle with *E. coli* O157 in Scotland was epidemiologically linked to contact with wild geese (501), and the organism was isolated from duck feces in an outbreak in people that followed swimming in a lake (457).

Poultry, especially pigeons in certain geographic areas, are a natural reservoir for Shiga toxin producing *E. coli* (STEC), which is a potential health hazard to people (104, 189, 351, 461). Pigeons may be infected with STEC but show no clinical disease (104, 351, 461, 484). Pigeon STEC produce the toxins Stx1, Stx2, and a variant of Stx2 identified as Stx2f, which is considered to be specific for pigeons (189). Stx2f is only weakly detected by conventional immunoassays (461). Clonally related, Shiga toxin-producing *E. coli* infected between 6–16% of urban

The authors wish to acknowledge the contribution of Dr. W. B. ("Bernie") Gross to this chapter in previous editions of *Diseases of Poultry* and to recognize his pioneering work on this disease in poultry.

pigeons in Rome. Infection was significantly higher in young pigeons compared to older pigeons (17.9% vs. 8.2%) (351). Shiga toxin producing enterohemorrhagic *E. coli* O128 were isolated from an 11-month-old child and 5 pigeons from the same area. Isolates were indistinguishable by molecular analyses (484). In poultry 52 of 97 APEC isolates from lesions of avian cellulitis, colisepticemia, swollen head syndrome, or diseased turkeys, along with 5 fecal isolates from healthy chickens, had Shiga toxin gene sequences. Most had *stx1*; only 3 had *stx2* (403). In contrast, pigeons in Colorado (406), chickens and pigeons in India (532), and chickens, pigeons, and gulls in Finland (277) were not a source of STEC.

Functional receptors for heat-stable enterotoxin occur throughout the avian intestinal tract (282). However, serotypes associated with diarrheal disease in humans and strains that produce both heat-labile and heat-stable enterotoxins have been isolated infrequently from chickens (5, 52).

Serotypes, virulence factors, and antimicrobial resistance of APEC in other animals are often shared. Avian strains potentially can be a source of genes and plasmids that encode for antimicrobial resistance and virulence factors (1, 339, 342, 352, 357, 435, 439) (see Virulence Factors below). APEC often share multiple virulence factors that are also commonly found in human uropathogenic *E. coli* and APEC plasmids can contribute to uropathogenicity of *E. coli* in a murine model of human disease (475). Poultry products can be a source of *E. coli* for people. Fresh poultry products, especially turkey, in grocery stores were frequently contaminated with *E. coli* that contained virulence and antimicrobial resistance factors (250, 251). An unexpected finding in a current study (L. Nolan, unpublished data) was that the majority of *E. coli* isolates from retail poultry products was more consistent with APEC than commensal *E. coli.* Additionally these APEC have considerable similarity to human uropathogenic strains suggesting the possibility that organisms causing urinary tract infections in people could potentially come from contaminated poultry products.

Salmonella enterica subsp. *enterica* serovar Newport, a disease-producing organism in people, and another *E. coli* serotype readily acquired antibiotic resistance in the absence of antibiotic selection pressure through transfer of a large conjugative resistance plasmid from antibiotic-resistant *E. coli* in the intestines of turkey poults. The plasmid was transferred to over 25% of *Salmonella* serovar Newport strains following co-infection (418). Antibiotic resistance of fecal *E. coli* was greater in broilers and turkeys that received antibiotics relatively frequently compared to layers, which had little exposure to antibiotics (517). Similar antibiotic resistance patterns were present in *E. coli* isolated from people who worked with these birds, and in some instances specific strains were shared among the birds and workers indicating that transmission of resistant organisms and/or plasmids from poultry to people occurs commonly. Resistance of chicken *E. coli* isolates to antimicrobials used mainly in people was minimal (6, 517).

History

Mortality of fowls and isolation of a bacterium from heart, liver, and spleen that was consistent with *E. coli* was first reported by Lignieres in 1894 (400). Following experimental inoculation, the isolate was virulent for pigeons, variably virulent for chickens depending on dose and route of administration, and not virulent for guinea pigs or rabbits. Subsequently diseases in grouse, pigeons, swans, turkeys, quail, and additional chicken flocks associated with a similar organism were documented between 1894 and 1922 (400).

The first description of colisepticemia was published in 1907 based on chickens dying from a cholera-like disease while being transported. It was concluded that, "*Bacterium* (*Escherichia*) *coli* may, under certain conditions, take on the ability to leave the intestines, become virulent, and cause a septicemia in hens, especially if their resistance has been weakened by hunger, thirst, cold, or lack of good ventilation" (400).

Infectious enteritis characterized by birds "going light" (infectious asthenia) and paralysis from which *E. coli* could be isolated was described in 1923 (400). In 1938 a pullorum-like disease caused losses of 15–40% in chicks less than 10 days of age that came from the same hatchery. The chicks had pericarditis, perihepatitis, and white spots on the liver. *E. coli* was isolated from tissues. Poor incubation resulting in weak chicks was identified as the reason for their susceptibility to infection (95).

Between 1938 and 1965, coligranuloma (Hjarre's disease) and the role of *E. coli* in a variety of lesions including air sac disease, arthritis, plantar abscesses (bumblefoot), omphalitis, panophthalmitis, peritonitis, and salpingitis were identified and described. Presence of *E. coli* in eggs (152) and *E. coli* infections following vaccination or natural virus infections were also documented (480).

Etiology

The etiology of colibacillosis is *Escherichia coli*. Other infectious agents and noninfectious factors usually predispose a bird to infection or contribute to the severity of the disease.

E. fergusonii is a closely related species that has been isolated from turkeys (137). Colicins (antibacterial substances) produced by *E. fergusonii* are similar to those produced by *E. coli* (477). *E. fergusonii* caused acute death in adult ostriches after a short period of anorexia, prostration, and severe hemorrhagic diarrhea. Lesions consisted of fibrinonecrotic typhlitis with intralesional colonies of Gram-negative bacteria (210).

Classification

Escherichia is the type genus of the family Enterobacteriaceae, which is composed of organisms that can grow aerobically or anaerobically and utilize simple carbon and nitrogen sources (45, 134). *E. coli* is the type species of the genus *Escherichia.* Additional species have been assigned to the genus but *E. coli* occurs most commonly and is most important as a pathogen. Although *Shigella* is still recognized as a genus with 4 species, they actually are *E. coli* strains (298, 543).

Name and Synonyms

Escherichia coli was initially named *Bacterium* (*Bacillus*) *coli commune*, which was shortened and modified to *B. coli* before

Table 18.1. Diagnostic characteristics of *Escherichia coli* and *E. fergusonii* (indicated as *E.f.*).

Gram-negative	
Rod (bacillus) shape	
Non-spore-forming	
MacConkey agar	(+) Pink colonies, precipitate *E.f.* clear colonies
Tergitol-7 agar	(+) Yellow colonies, *E.f.* red colonies
EMB agar	(+) Dark colonies, metallic sheen
Motility	(v)
Catalase	(+)
Oxidase	(–)
Nitrates -> nitrites	(+)
Gelatin	(–)
Hydrogen sulfide	(–)
Indole	(+)
Methyl red	(+)
Voges-Proskauer	(–)
Citrate (Simmons)	(–)
Urease	(–)
KCN medium	(–)
Lysine decarboxylase	(+)
Ornithine decarboxylase	(v) *E.f.* (+)
Phenylalanine deaminase	(–)
Glucose	(+) Acid & gas
Lactose	(+) Infrequently (–); *E.f.* (–)
Mannitol	(+)
Dulcitol	(v)
Sucrose	(v) *E.f.* (–)
Salicin	(v)
Adonitol	(–) *E.f.* (+)
Inositol	(–)
Sorbitol	(+) *E.f.* (–)
Malonate	(–) *E.f.* (v)
Cellobiose	(–) *E.f.* (+)

(+) growth or reaction occurs
(–) growth or reaction does not occur
(v) reaction or character is variable among isolates

being given its present name by Castellani and Chalmers in 1919 (134). The genus is named for Theobald Escherich, a pediatrician who first identified and described the organism, which appeared in the feces of infants soon after they began nursing. It is typical of bacterial species within the family Enterobacteriaceae (59). Diagnostic characteristics of *E. coli* and *E. fergusonii* are presented in Table 18.1.

Morphology and Staining

Escherichia coli is a Gram-negative, non-acid-fast, uniform staining, non-spore-forming bacillus, usually 2–3 × 0.6 μm. Organisms grown in culture are more variable in size and shape. Intracellular organisms are often smaller than extracellular bacteria. Most strains are motile and have peritrichous flagella.

Growth Requirements

Escherichia coli grows aerobically or anaerobically on ordinary nutrient media at temperatures of 18–44°C. It ferments carbohydrates, often producing gas. Generation time and number of organisms during a specific time period are related to temperature (see Table 18.2).

Table 18.2. Effect of temperature on generation time and numbers of *Escherichia coli* that could develop within 24 hours in the absence of limits on growth (nutrition, accumulation of inhibitory substances, etc.).

Temperature		Generation	
(°F)	(°C)	Time (hours)	No. of *E. coli* in 24 hours
32	0	20	2
40	4.4	6	8
50	10.0	3	128
60	15.6	2	2,048
70	21.1	1	8,388,608
80	26.7	0.75	3,435,973,800
90	32.2	0.50	24,073,749,000,000
100	37.8	0.30	236,118,320,000,000,000,000

Colony Morphology

On agar plates incubated for 24 hours at 37°C, colonies are low, convex, smooth, and colorless. Colonies are bright pink and surrounded by a precipitate on MacConkey's agar, have a dark green-black metallic sheen on eosin-methylene blue (EMB) agar, and are yellow on tergitol-7 agar. Although colony morphology may vary they are usually 1–3 mm in diameter with granular structure and an entire margin. Rough colonies are larger with irregular margins. Mucoid colonies are raised, larger, appear wet, and are sticky when probed. In contrast to the frequent occurrence of hemolysis by mammalian pathogenic *E. coli* on blood agar, hemolysis is not a common characteristic of APEC. *E. coli* rapidly produces diffuse turbidity in broth cultures.

Biochemical Properties

Acid and gas are produced from fermentation of glucose, maltose, mannitol, xylose, glycerol, rhamnose, sorbitol, and arabinose, but not dextrin, starch, or inositol. Substituting sorbitol for lactose in MacConkey agar is useful for distinguishing *E. coli* O157:H7 from other *E. coli* because O157:H7 typically does not ferment sorbitol and will not appear as pink colonies in contrast to typical *E. coli* isolates. Most *E. coli* isolates ferment lactose, but negative strains, which must be differentiated from *Salmonella*, are occasionally isolated. Fermentation of adonitol, sucrose, salicin, raffinose, and dulcitol is variable. Isolates that fermented raffinose and sorbose produced high mortality in an embryo lethality test (349). *E. coli* produces indole, a positive methyl red reaction, and reduces nitrate to nitrite. Voges-Proskauer and oxidase reactions are negative and hydrogen sulfide is not produced on Kligler's iron medium. *E. coli* does not grow in the presence of potassium cyanide, hydrolyze urea (urease negative), liquefy gelatin, or grow in citrate medium. Biochemical tests can be used to distinguish *E. coli* from other *Escherichia* species (45) and bacteria in the family Enterobac-

teriaceae (134). *E. fergusonii* does not ferment lactose, sucrose, raffinose, or sorbitol, which helps distinguish it from *E. coli* (Table 18.1).

Susceptibility to Chemical and Physical Agents

Escherichia coli possess no unique resistance capabilities and have a susceptibility pattern to chemical and physical agents typical of vegetative, Gram-negative bacteria. Inactivation of most strains will occur at temperatures ranging from 60°C for 30 minutes to 70°C for 2 minutes. Thorough pre-cleaning and/or presence of a germicide enhance thermal inactivation. The organism survives freezing and persists for extended periods at cold temperatures. Thermal inactivation in litter to achieve a 90% reduction in the number of bacteria is dependent on time and temperature ranging from 1–2 days at 37°C to 6–22 weeks at 4°C. Inactivation in litter is slower in the presence of high moisture and more rapid when free ammonia is present (213).

Reproduction of most strains is inhibited by a pH of less than 4.5 or greater than 9, but the organism is not killed. Some virulent strains, *e.g.*, O157:H7, are acid tolerant, which permits them to pass through the stomach without being killed. Organic acids are more effective than inorganic acids at inhibiting growth. Treatment with citric, tartaric, or salicylic acids significantly reduces coliform counts in poultry litter (240). A salt concentration of 8.5% prevents growth but does not inactivate the organism (41).

Stabilized chlorine dioxide is highly effective when used as a water disinfectant (405). Chlorate in feed selectively reduces the number of *E. coli* and related bacteria in the digestive tract by converting relatively nontoxic chlorate to highly toxic chlorite via the same pathway *E. coli* uses to convert nitrate to nitrite (14). Solar disinfection of water through the action of ultraviolet light and temperature is a low-cost method of treating drinking water for people in developing areas to reduce or eliminate enteric bacteria that may have application in the poultry industry (42).

Drying is detrimental to the organism. When samples of flooring from broiler transport coops were contaminated with *E. coli* and allowed to dry for 24 or 48 hours, only very few organisms were still viable (43). Washing before drying completely eliminated the organism.

Resistance to Metals and Disinfectants

Escherichia coli has the ability to acquire resistance to a broad range of heavy metals (arsenic, copper, mercury, silver, tellurium, zinc) and disinfectants (chlorhexidine, formaldehyde, hydrogen peroxide, quaternary ammonium compounds). Specific strains can vary substantially in their susceptibility to heavy metals and disinfectants (2, 458). Strains develop resistance to disinfectants when subjected to environmental selection pressures. Limited studies have indicated that resistance to disinfectants is not widespread or common in *E. coli* infecting animals (2), but it is transferable via mobile genetic elements and will likely become more frequent (34). In one study, no resistance to hydrogen peroxide or formaldehyde was found (2). Formaldehyde resistance in *E. coli* is based on a plasmid mediated DNA fragment known as the formaldehyde resistance gene, which encodes for formaldehyde dehydrogenase (285).

Plasmids that provide resistance genes for antibiotics also often provide resistance genes for disinfectants and heavy metals (255, 258). In addition to antibiotic resistance, the APEC IncHI2 plasmid, p-APEC-O1-R, conferred resistance to potassium tellurite, silver nitrate, copper sulfate, and benzalkonium chloride following transfer of the plasmid to a recipient strain by conjugation (259). Similarly the APEC IncF plasmid, pAPEC-O2-R also encoded for resistance to quaternary ammonium compounds and silver and other heavy metals, as well as several antibiotics (256).

Antigenic Structure and Toxins

Serotypes of *E. coli* are classified according to the Kauffmann scheme (134). At present there are approximately 180 O, 60 H, and 80 K antigens (490); the numbers change as new ones are identified and previous ones that are duplicated or attributable to another bacterial species are removed. In most serologic typing schemes only the O and H antigens are determined, e.g., O157:H7. The O antigen determines serogroup while the H antigen determines serotype. Rough strains autoagglutinate and cannot be serotyped. Additional serotypes with O antigens that have not been recognized also are found in most surveys. Fimbrial (pilus) antigens are included in serotyping when considered important.

O (Somatic) Antigen

Lipopolysaccharide (LPS) in the cell wall, also known as endotoxin, is a polysaccharide-phospholipid complex that is released when the cell undergoes lysis. O antigen is the antigenic portion of LPS whereas the toxic portion of the molecule is lipid A. O antigen is resistant to boiling. Methods to prepare and use antisera, which typically agglutinate O antigen at high titers (usually over 1:2560) when antigen-antibody mixtures are incubated at 50°C for 24 hours, have been described (548).

H (Flagellar) Antigen

To examine for H antigens, isolates must be grown under conditions that promote motility. H antigens are proteins found in the different types of flagellin that comprise the flagella. Heating to 100ºC destroys them. Tube agglutination tests are read after incubation at 50°C for 2 hours (548).

K (Capsular) Antigen

K antigens are polymeric acids containing 2% reducing sugars, are associated with virulence, are on the surface of the cell, interfere with O agglutination, and can be removed by heating for 1 hour at 100°C. A few strains require heating for 2.5 hours at 121°C. On the basis of heat stability, K antigens are subdivided into L, A, and B forms. Antisera are prepared in rabbits by inoculating live organisms intravenously. Tube agglutination titers are determined by incubating antigen-antibody mixtures at 37°C for 2 hours and overnight at 4°C. Titers are low (1:100–1:400). Most of these antigens can be identified by the slide agglutination test using appropriately diluted serum (548). Presently K antigens are not commonly included in serotyping.

F (Pilus) Antigen

F antigens are involved in attachment to cells. They are variably expressed depending on the environment in which the organism is growing both *in vitro* and *in vivo*. Pili are classified as being mannose sensitive or mannose resistant depending on whether or not agglutination is inhibited or unaffected respectively when mannose is present. A variety of tests have been developed for detecting fimbrial antigens (548).

Toxins

APEC are much less toxigenic than pathogenic *E. coli* in mammals and human beings. While APEC do not commonly produce enterotoxins, other toxins are elaborated but their roles in diseases of poultry are currently uncertain (see Virulence Factors Toxins). Pigeons can be a significant source of Shiga-toxin producing *E. coli* strains (see Public Health Significance).

Strain Classification

Antigenicity

Even though molecular methods for identifying specific virulence genes are available, serotyping remains a useful tool for epidemiologic studies. Serotyping provides a means of relating previous work with new work. Also it is important to know the serotype of an APEC strain because the immune response in poultry primarily is directed against O antigens. Numerous surveys have been made in many parts of the world to determine serotypes most frequently associated with diseases in poultry caused by *E. coli* (440, 466, 507). Variations according to geographic region occur, but in most studies the common serotypes have been O1, O2, O35, O36, and O78 (208, 480). Many other serotypes have been found less frequently, while some APEC do not belong to known serotypes or are untypeable (561). Some outbreaks are consistently associated with a specific serotype, *e.g.*, O111 causing mortality, septicemia, and polyserositis in egg-laying chickens (560).

Sixty-two different O types were found among typeable strains in a study comparing serotypes of 458 *E. coli* isolates from chickens with colibacillosis to 167 isolates from healthy chickens. Only 15% of the strains belonged to the serogroups O1, O2, O35, O36, or O78, which previously had been associated with avian colibacillosis. Several isolates from diseased birds belonged to 5 serogroups (O18, O81, O115, O116, O132), which have not previously been associated with colibacillosis. Although serotypes from diseased birds were significantly different from ones from healthy birds, intestinal infection of healthy birds with serotypes isolated from diseased birds still occurred frequently (53).

Genetic or Molecular

In addition to phenotyping and serotyping, isolates of *E. coli* can be further characterized by antibiotic resistance, toxigenicity, presence of adhesins including piliation or other virulence factors, cell attachment, hemagglutination, lysogeny (phage typing), and plasmid profiling. DNA probes and polymerase chain reactions (PCRs) have been developed to detect specific genes important in virulence (281, 291, 314, 548). Detection of multiple genes that encode virulence factors by multiplex PCR is an efficient method for identifying several characteristics of APEC and commensal strains simultaneously (133, 436, 474).

Methods of "fingerprinting" isolates including pulsed-field gel electrophoresis (PFGE), amplified fragment length polymorphism (AFLP), restriction fragment length polymorphism (RFLP), and random amplification of polymorphic DNA (RAPD) can be used in epidemiological studies (252, 330). PFGE permitted fingerprinting of *E. coli* isolates from chickens with cellulitis. Specific types of the organism were found consistently associated with farms and successive flocks (471, 472).

RAPD is a rapid, cost-effective procedure for determining clonal types of *E. coli* in epidemiologic studies (71, 330). It is less costly and quicker than molecular fingerprinting using RFLP (330). RAPD analysis of 55 isolates revealed 50 subtypes in three clusters. It was not helpful for discriminating between pathogenic and nonpathogenic isolates (71). Sixteen different RAPD types were identified in a collection of isolates from diseased and healthy poultry in Georgia. Differences in the types were discovered but, with the exception of one RAPD type that occurred only in diseased chickens and accounted for 23% of the isolates, the differences were not absolute. Also, RAPD types did not correlate with antibiotic resistance profiles (330).

Multilocus enzyme electrophoresis (MLEE) identified specific genotypes, which demonstrated that relatively few clonal types are responsible for different forms of colibacillosis in chickens and turkeys in widespread geographic areas. Virulence varied little among isolates within a clonal group but varied considerably between clonal groups (539). MLEE applied to a large number of *E. coli* isolates showed that they could all be placed into one of 4 clonal groups designated A, B1, B2, and D. Several clonal groups were identified among isolates from chickens with omphalitis, swollen head syndrome, septicemia, and intestines of healthy chickens in Brazil. MLEE permitted better discrimination of the isolates than ribotyping; most pathogenic isolates clustered together in two clonal subgroups while most commensal isolates occurred in other clonal groups (94).

Assignment of APEC to phylogenetic typing can also be accomplished with a multiplex PCR-based method (81). This method assigns isolates to A, B1, B2, and D phylogenetic groups. Clonal groups B2 and D are considered to contain most of the pathogenic isolates. However, Rodriguez-Siek *et al*. (435) found that the majority of 524 APEC isolates fell into the nonpathogenic clonal groups. MLST (multilocus sequence typing) also can be used to construct phylogenetic trees, providing insight into the evolution of each of these clones. Highly virulent strains tend to be hybrids that evolved relatively recently by homologous recombination (543).

Genomes of *E. coli* contain multiple sequences, which can be identified by PCR and used to characterize individual isolates of the organism and determine their relatedness to each other. The procedure is known as enterobacterial repetitive intergenic consensus (ERIC). ERIC can be combined with repetitive extragenic palindromic (REP) PCR, which determines repetitive sequences outside of the genome. Using these methods dendrograms based on the different patterns revealed extensive genetic diversity among avian *E. coli* strains. Pathogenic and nonpathogenic iso-

lates tended to group in different clonal groups whereas serotypes were distributed among all groups. No specific genotype or serotype could be identified as being the cause of colibacillosis (99). In a subsequent study commensal and omphalitis isolates grouped together while APEC from poultry with septicemia or swollen head syndrome occurred in different clonal groups indicating the opportunistic nature of the omphalitis isolates (93).

Signature-tagged transposon mutagenesis (STM) (308) and genomic suppression subtractive hybridization are methods that have been useful in identifying previously unknown putative virulence genomic sequences in APEC (61, 268, 342, 462, 491). The newly recognized genetic sequences did not occur in commensal strains suggesting their importance in virulence. Mutant strains lacking the putative novel virulence factors were less virulent than the APEC strains from which they originated. Furthermore, these novel virulence factors were more frequent in other avian and mammalian pathogenic strains than commensal strains. The specific nature of the genes and their role in the pathogenesis of colibacillosis in poultry remain to be determined.

When two virulent avian strains (O2 and O78) from colisepticemia cases were compared, they were found to be very different with only a few shared genes. Analysis of additional strains of each serogroup of human and animal origin showed similar genetic diversity leading to a "mix-and-match" theory, *i.e.*, different virulence factors can combine to provide an ability to cause septicemia (342).

Presence of *E. coli* in water indicates fecal pollution since the organism does not normally live outside of the intestinal tract and is present in high numbers in feces. To determine the source of the pollution, *E. coli* isolates can be classified as to species of origin by ribotyping (66, 190). Riboprints of fecal *E. coli* provided the most accurate determination of source when comparisons were made among no more than three. For example, the source of *E. coli* from geese, turkeys, or chickens was correctly determined for approximately 96% of the isolates by the procedure (66).

Pathogenicity

Ability to cause mortality in embryos or chicks differentiates APEC from commensal *E. coli* strains (158, 159, 160, 349, 392). An embryo lethality test can be used to test avian *E. coli* isolates for virulence. Eleven 12-day-old chicken embryos were inoculated via the allantoic cavity with 100 cfu of the test organism. Two-day mortality was <10% for nonvirulent strains, 10–29% for intermediate strains, and >29% for virulent strains (547). Extending the post-inoculation observation time resulted in higher mortality, but the pattern of mortality among various strains remained essentially unchanged (349). Intravenous and subcutaneous inoculation of chicks correlated with embryo lethality, whereas intratracheal inoculation did not (160). Compared to the embryo lethality test, virulence of an isolate correlated with complement resistance and presence of ColV plasmid, but neither of these tests conclusively identified all isolates as virulent strains. The embryo lethality test remains the best single test for discriminating APEC from commensal *E. coli* (158).

Virulence Factors

It is widely believed that avian colibacillosis is a secondary disease and that APEC, the etiologic agents of colibacillosis in birds, are opportunists. However, increasing evidence has shown that clones of APEC exist that are especially well adapted to life as pathogens, suggesting that APEC infections might not always be opportunistic or secondary to some predisposing condition. Certainly, APEC, like other pathogenic *E. coli*, have acquired genes by horizontal transfer that encode virulence factors, which serve to distinguish APEC from commensal strains (111, 436, 491). These virulence genes may be clustered into chromosomal- or plasmid-located pathogenicity islands (PAIs). Acquisition of virulence genes, plasmids, and/or PAIs enables APEC to survive within the host, which can result in clinical disease. Since APEC usually cause extraintestinal disease, they are commonly classified into a group of *E. coli* known as extraintestinal pathogenic *E. coli* or ExPEC (263, 449). ExPEC also include uropathogenic *E. coli* (UPEC) and meningitis-associated *E. coli* (MNEC) that cause disease in human beings and other hosts (263). ExPEC share certain virulence attributes enabling their extraintestinal lifestyle, including adhesins, toxins, protectins, iron acquisition mechanisms, and invasins (263, 435, 436). Identification of such traits among APEC has fostered development of a rudimentary definition of an APEC pathotype (436) and has led to interest in the potential of APEC to infect non-avian hosts (357, 435, 439).

Despite the fact that most APEC infections are extraintestinal, some APEC contain traits associated with the intestinal *E. coli* pathotypes, including enteropathogenic *E. coli* (EPEC) (264, 291), enterotoxigenic *E. coli* (ETEC) (264, 292), enteroinvasive *E. coli* (EIEC) (440, 441), and enterohemorrhagic *E. coli* (EHEC) (292, 484). Furthermore, APEC strains causing the same disease may differ substantially in their gene content (342). In view of this high level of genomic plasticity, it is not surprising that no single virulence factor has been identified that will distinguish all APEC from all commensal *E. coli* strains. Genes encoding virulence factors in APEC are summarized in Table 18.3.

Adhesins

Adhesins may be fimbrial or nonfimbrial. The role of fimbriae in the pathogenesis of avian colibacillosis is unclear, although it seems likely that these virulence factors would be important in APEC's colonization of the host (23). Fimbriae can undergo phase variation depending on the types present on the organism and tissue being colonized. Several fimbrial types have been described among APEC, including AC/I (avian *E. coli* I) (23, 386, 555), P (F11) (292), type 1 (F1) (17, 292), Stg (316), and curli (172, 287, 291). Also, a ColV plasmid, encoding type 4 pili, has been found in an APEC O78 strain. Although type 4 pili are known to contribute to host cell adherence of some bacteria, their roles in APEC adherence and virulence, if any, are unknown (174).

F1 fimbriae are expressed during initial colonization of tracheal epithelial cells, whereas P fimbriae are expressed later when the organism is in the lower respiratory tract or body tissues. Bacteria are rapidly killed by macrophages when they express F1 fimbriae (420, 422). Although the F1 fimbrial adhesin, FimH, is required for adhesion to cultured chicken epithelial pha-

Table 18.3. Putative APEC virulence and virulence-associated genes and regions. Modified from 248, 263, 436.

Gene, Operon or Region	Description
Iron-Related Genes	
feoB	Primary gene mediating ferrous (Fe^{2+}) iron uptake
ireA[1]	Iron regulated, siderophore receptor, outer membrane protein
Yersiniabactin operon[1]	
irp2	Encodes iron repressible gene associated with yersiniabactin synthesis
fyuA	Encodes ferric yersiniabactin uptake receptor
Sit operon[1,2]	
sitA	Putative iron transport operon
Aerobactin operon[2]	
iutA	Ferric aerobactin outer membrane receptor gene
iucC	Involved in aerobactin synthesis Aerobactin operon is involved in iron uptake and transport
Salmochelin operon[2]	
iroN	Catecholate siderophore receptor gene
Eit operon[2]	
eitA	ABC iron transporter; periplasmic-binding protein
Toxin/Bacteriocin-Related Genes	
stx1, stx2[3]	Shiga toxins; inhibit protein synthesis
hlyD	Transport gene of the α-hemolysin operon
hlyF[2]	Avian *E. coli* hemolysin
cdtB	Cytolethal distending toxin; DNase I activity; blocks mitosis
vat[1]	Vacuolating autotransporter toxin
cnf1	Cytotoxic necrotizing factor 1; altered cytoskeleton, necrosis
usp	Uropathogenic-specific protein (bacteriocin)
ColV operon[2]	
cvaC	Structural gene of the ColV operon
ColB operon[2]	
cbi	Immunity gene of the ColB operon
ColM operon[2]	
cma	Structural gene for ColM activity
Adhesins	
Type 1 fimbrial adhesin operon (Fim)	
fimH	D-mannose-specific adhesin of Type 1 fimbriae
Pap pilus operon[1]	
papC	Acts as a molecular usher in P pilus assembly
papA	Encodes the major structural subunit
papG	Encodes the pilus tip adhesin
S fimbrial operon S fimbrial family of adhesins differ in receptor specificity S fimbriae (Sfa)	
sfaS	Encodes pilus tip adhesin of S fimbriae; S fimbriae interact with glycoproteins containing sialic acid
F1C-fimbriae (Foc)	
focG	Encodes a component of F1C fimbriae
focA	Encodes the major fimbrial subunit
S/F1C-related fimbriae (Sfr)	
AC/I fimbriae (Fac)	
facA	Encodes major subunit of avian *E. coli* I (AC/I) fimbriae
Curli operon	
crl	Gene cluster encoding for curli fibers; involved in cell adhesion and internalization
iha	IrgA homologue adhesin
afa	Afimbrial adhesin, a member of the Dr family of adhesins
gafD	G fimbrial adhesin
bmaE	Blood group M-specific adhesin
Stg operon	
stgA	On the C-terminus of the Stg fimbrial operon
tsh[2]	Temperature-sensitive hemagglutinin gene
bfp	Bundle-forming pilus, initiates attaching and effacing lesions in typical AEEC isolates
eae	*E. coli* attaching and effacing gene that encodes intimin
Protectins	
iss[2]	Encodes an outer membrane protein involved in increased serum survival and surface exclusion
traT[2]	Encodes an outer membrane protein involved in serum resistance and surface exclusion
bor	Virulence determinant encoded by λ bacteriophage; involved in serum resistance
ompA	Outer membrane protein A is involved in serum resistance
kps cluster	Involved in encoding capsular (K) antigens
Invasins	
ibeA[1]	Promotes invasion of brain microvascular endothelial cells
ipa	Cell penetration and intracellular survival
tia[1]	Tia invasion determinant
Miscellaneous	
ompT[1,2]	Encodes a protease able to cleave colicin
malX	Pathogenicity island marker from UPEC CFT073
fliC (H7)	Produces flagellin protein associated with the H7 antigen group
Ets operon[2]	
etsA	Encodes ABC transporter, efflux pump protein

[1]localized to an APEC chromosomal PAI
[2]localized to APEC plasmid-linked PAIs
[3]encoded by phage

ryngeal or tracheal cells, lack of FimH favors *in vivo* colonization of the trachea of chickens (17). Curli may contribute to bacterial invasion of eukaryotic cells (172) and APEC's persistence in the cecum (287).

Intimin is a non-fimbrial adhesin encoded by the *E. coli* attaching and effacing (*eae*) gene, which is found in EHEC and EPEC. It permits the bacterial cell to adhere to the surface of the enterocyte, which initiates a characteristic attaching and effacing (AE) lesion. Several genetic variants of intimin, identified by letters of the Greek alphabet, have been identified. The most common type in APEC is ß-intimin followed by λ-intimin (281, 492). A highly virulent APEC (O86:K61), which has caused mass mortality of passerine birds in Britain, produces λ-intimin (291).

Organisms producing AE lesions are known as attaching and effacing *E. coli* (AEEC). In mammals a specific pilus (bfp, bundle-forming pilus) occurs together with intimin to cause the AE lesion. AEEC that do not have bfp are referred to as "atypical AEEC." Avian AEEC are usually atypical AEEC as isolates infrequently have bfp. AEEC are either absent or found infrequently in most surveys of poultry (281, 492) except for pigeons infected with Shiga toxin strains (189, 484). However, high percentages of *eae*+ isolates were obtained from dead-in-shell embryos and chicks with yolk sac infections in Mexico (30%) (440) and feces of healthy chicks in Kenya (60%) (264), suggesting there may be certain geographic areas where AEEC commonly infect chickens.

A novel avian respiratory soluble lectin, distinct from pulmonary collectins and ficolins, that binds with surface polysaccharides of pathogenic *E. coli* (serogroups O2 and O78) has been discovered in air sac fluids of turkeys. Its role, if any, in colibacillosis has yet to be defined (534). Also, temperature-sensitive hemagglutinin (Tsh), the first described serine protease autotransporter of the Enterobacteriaceae (SPATE) (424), is secreted by some APEC strains. It is a bifunctional protein that acts as an adhesin and protease (279) and mediates colonization of the host's respiratory tract during early infection (116). The contribution of Tsh to the pathogenesis of colisepticemia appears to be restricted to the early stages of infection, as *tsh*⁻ mutants cause less severe and less frequent lesions in air sacs. Tsh does not appear to be required for high levels of APEC virulence (510). Reports of its prevalence among different APEC populations vary widely (9, 98, 102, 103, 132, 133, 243, 329, 335, 435, 436, 523, 553, 561).

Toxins

APEC tend to be less toxigenic than mammalian pathogenic *E. coli* (52, 243, 337). This difference may be due to the lack of toxin production or that toxins produced by avian strains are not detectable with tests for toxins produced by mammalian strains. In addition to endotoxin, a structural component of the organism's cell wall, APEC can elaborate several toxins that are important in disease (402, 403, 454, 455, 456); low level occurrence of certain toxin genes among APEC has been reported. These genes include those encoding cytolethal distending toxin (292, 435, 436, 440), cytotoxic necrotizing factor 1 (435, 436) and various hemolysins (9, 257, 258, 335, 352, 365, 430, 435, 436, 549).

Some of the genes encoding toxins occur in a substantial number of APEC. A *Salmonella* virulence homologue, *hlyF*, was first described in an avian *E. coli* isolate in 2004 (352). It shares significant homology with the *E. coli* K12 "silent" hemolysin gene *she* and occurs commonly among APEC. The gene is found within a virulence cluster of large, conjugative ColV and ColBM plasmids (257, 258). Its role in APEC virulence is not yet known.

Vacuolating autotransporter toxin (Vat), which is encoded by the *vat* gene, also occurs commonly among APEC (132). Vat is a 148.3-kDa protein, which has a structure typical of SPATE. It causes cytotoxic effects in cultured cells similar to those caused by *Helicobacter pylori* VacA toxin. Vat appears to be a virulence factor for APEC, as deletion of the *vat* gene results in attenuation of virulence (404).

Iron Acquisition Mechanisms

The ability of APEC to obtain iron is well documented and likely due to various iron-acquisition mechanisms (aerobactin, yersiniabactin, *sit*, and *iro* systems) (110, 117, 173, 296, 451, 544). Genes of these operons occur frequently among APEC, but are significantly less common in commensal *E. coli* strains (436). APEC frequently contain several of these operons, one or more of which may be found on large plasmids (112, 117, 162, 237, 254, 258, 259, 435, 436, 451, 510). This apparent redundancy in iron acquisition mechanisms and widespread distribution of these systems among APEC suggest that the ability to obtain iron is important in the pathogenesis of avian colibacillosis.

The *sit* operon was originally described in a *Salmonella enterica* serovar Typhimurium isolate (563) and more recently identified in APEC using genomic subtractive hybridization and signature-tagged mutagenesis (308, 462). The *sit* operon encodes an ABC transport system involved in metabolism of iron and manganese and resistance to hydrogen peroxide (451). In at least one APEC, this operon occurs in both chromosomal and plasmid-located PAIs (257). In its plasmid location, *sit* is closely associated with the aerobactin siderophore operon and *iro* locus (257). The yersiniabactin operon in at least one APEC is found in a chromosomal PAI (GenBank accession no. NC 008563).

Protectins

Ability to resist complement is a common characteristic of APEC, regardless of the syndrome or avian host species of origin (393). Resistance of *E. coli* to complement is related to several structural factors including K1 capsule (89, 90, 154, 338) or other capsule type (448, 494), a smooth lipopolysaccharide (LPS) layer (90, 167) or particular LPS type (338), and certain outer membrane proteins (OMPs), including TraT, Iss, and OmpA (47, 78, 79, 343, 537). When 294 APEC were compared with 75 fecal *E. coli* isolates from clinically healthy birds for possession of a capsule, smooth LPS layers, *ompA*, *traT*, and *iss*, only *iss* was found to occur significantly more often in APEC relative to commensal strains (414).

The increased *s*erum *s*urvival gene (*iss*), first described by Binns and coworkers in 1979 for its role in complement resistance associated with a ColV plasmid, increased the virulence of an *E. coli* 100-fold for day-old chicks (46) and its complement

resistance over 20-fold (31, 78, 79). The gene *iss* encodes Iss, a lipoprotein exposed on the outer membranes of *E. coli* (318). It occurs frequently among APEC (435, 436, 553, 561) compared to a low rate of occurrence in commensal strains. Although it has been proposed that the substantial difference in distribution of *iss* between APEC and commensal strains might reflect its importance in APEC virulence (393), Mellata *et al*. (338) reported that *iss* did not play a major role in resistance of APEC strain χ7122 to serum. In contrast, Tivendale *et al.* (510) found a strong association between APEC virulence and carriage of *iss* and/or *iucA*, a gene of the aerobactin operon. From such disparate observations it is evident that much remains to be learned about complement resistance in APEC, its mediators, and its role in disease pathogenesis.

The ability of APEC to resist the detrimental effects of heterophils and macrophages is likely another important determinant that contributes to successful infection. Resistance to phagocytosis or its effects may be related to complement resistance or possession of other traits. Kottom *et al*. (280) reported that a complement-sensitive mutant bound significantly more C3 subunits and was phagocytosed significantly more often than the wild-type APEC strain from which it had been derived. It was hypothesized that the mutant's decreased virulence resulted from its increased sensitivity to complement-mediated bacteriolysis or enhanced susceptibility to complement-opsonized phagocytosis. However, subsequent studies by Mellata *et al*. (338) showed that non-opsonized APEC were eliminated by phagocytes to the same or greater extent than serum-opsonized bacteria.

Phagocytosis of APEC by avian phagocytes is promoted by presence of type 1 fimbriae and absence of P fimbriae, K1 capsule, O78 antigen, and an uncharacterized pathogen-specific chromosomal region. Presence of type 1 and P fimbriae, O78 antigen, and the 0-minute chromosomal region contributed to protection of APEC against the bactericidal effect of phagocytes, in particular, heterophils (338).

Certain strains of APEC can survive within macrophages and cause their destruction through apoptosis (35, 434). Caspases, enzymes essential for apoptosis, were activated by a strain of APEC (APEC17), which resulted in cytotoxicity within 8 hours of infection (35).

Invasins

The *ibeA* gene contributes to invasion of brain microvascular endothelial cells (BMEC) by neonatal meningitis ExPEC. It is significantly more likely to be found in APEC than in avian commensal strains (156, 268, 435, 436). The abilities of APEC strain BEN 2908 to invade human BMEC and also cause avian colibacillosis are significantly reduced when *ibeA* is inactivated (156). These results indicate that *ibeA* is a virulence attribute of APEC. The gene *ibeA* occurs in between 14% and 20% of APEC (156, 435, 436). In at least one APEC strain, *ibeA* is found in a chromosomal PAI (GenBank accession no. NC 008563).

Other

Formation and residence within a biofilm could enhance the ability of APEC to resist cleaning and disinfection and to acquire virulence and resistance genes by horizontal gene transfer. When 105 APEC and 103 avian commensal *E. coli* strains were compared for their ability to form biofilms on plastic surfaces, formation of biofilms by APEC was induced by nutrient poor conditions. In contrast commensals formed biofilms in both nutrient poor and rich conditions (476).

Genomic Location of Virulence Genes

Much progress has been made on localizing various virulence genes in the APEC genome, providing insight into their organization, regulation, and evolution. Such progress is expected to accelerate with recent completion of the genomic sequence of an APEC strain (GenBank accession no. NC008563). Sequences of several APEC chromosomal PAIs and two APEC virulence plasmids are currently available. Both virulence plasmids contain PAIs (257, 258). During conjugation, these virulence plasmids transfer from donor to recipient strains with large multi-drug resistance-encoding plasmids (256, 259), and genes encoding virulence and resistance can be found on the same APEC plasmids (personal communication with Timothy J. Johnson). This close association between resistance and virulence genes in APEC may offer a means by which APEC persist in the production environment (255).

Common features of most PAIs include encoding one or more virulence factors, size between 10–200 kb, introduction into the genome via horizontal transfer, which may result in deviation of G-C ratios and codon usage from the organism's typical pattern, and usually being flanked by small directly repeated sequences (262). PAIs may contain mobility elements, such as integrons, transposons, and insertion sequences. If they themselves move they are likely carried on plasmids, conjugative transposons, or phages, whose loss may spontaneously convert a virulent organism into an avirulent one (262).

Chromosomal PAIs identified among APEC include the VAT-PAI (404), PAI $I_{APEC\text{-}O1}$ (269), and AGI-3 (76). VAT-PAI is a 22-kb PAI that includes the *vat* gene, which encodes Vat (see Virulence Factors:Toxins). Another chromosomally located APEC PAI, PAI $I_{APEC\text{-}O1}$ (269), is 56 kb in size and harbors the complete *pap* operon and other *E. coli* genes (*tia* and *ireA*). Also, PAI $I_{APEC\text{-}O1}$ lies immediately upstream of the *kps* gene cluster, which is required for biosynthesis of polysialic acid capsule. Although the role of this PAI in virulence has yet to be elucidated, study of 95 APEC and 95 avian commensal isolates for possession of 6 genes of this PAI revealed that they occurred more often in APEC of high and intermediate virulence than in isolates of low virulence. None of the commensals contained all 6 of these targets; whereas, 7.2% of APEC strains had all of the genes (267). The most recently described APEC chromosomal PAI, AGI-3 (76), is 49.6 kb in size and is arranged in 5 modules. Deletion analysis of AGI-3's module 1 demonstrated that it contributed to an APEC strain's carbohydrate uptake and virulence for chickens. Studies of its prevalence among 249 ExPEC strains, including 205 APEC and 36 nonpathogenic strains of avian origin, showed that about 12% of all strains tested contained this region. All 15 APEC strains of the O5 serogroup contained this PAI, suggesting that it might be serogroup-associated.

APEC PAIs also have been found on large transmissible plasmids. A 180-kb ColV plasmid, known as pAPEC-O2-ColV, was sequenced and analyzed (258), and its role in virulence was evaluated (475). In addition to regions devoted to plasmid transfer, maintenance, and replication, pAPEC-O2-ColV contained a 94-kb cluster of putative virulence traits, including *hlyF*, *ompT*, *iss*, *tsh*, the ColV operon, and four putative iron-related systems. The iron-related systems included those encoding aerobactin and salmochelin, the *sit* ABC transport system, and a putative iron transport system novel to APEC called *eit*. Also, this PAI contained another putative ABC transport system known as *ets*. A study of the distribution of these PAI genes in 595 APEC and 199 avian fecal commensal *E. coli* isolates revealed that a portion of this PAI was highly conserved among APEC and that the genes of the conserved region occurred more often in APEC than in commensal strains. This conserved portion, which occurred in nearly 80% or more of APEC examined, included the *sit*, salmochelin, aerobactin, and *ets* operons; *hlyF*; *iss*; *ompT*; the RepFIB replicon; and the 5′ end of the ColV operon. The variable portion of this PAI contained the 5′ end of the ColV operon, *tsh,* and the *eit* operon. The split between conserved and variable portions occurred within the *cvaB* gene of the ColV operon with the 5′ end of *cvaB* and many of its upstream genes occurring significantly more often among APEC than the 3′ end of *cvaB* and many of its downstream genes. This difference in prevalence between conserved and variable portions of the PAI among APEC suggested that there must be an alternative location for the conserved portion in APEC. Indeed, a very similar PAI was found in a 174-kb ColBM-encoding APEC plasmid, known as pAPEC-O1-ColBM (257). This F-type plasmid shares remarkable similarities with pAPEC-O2-ColV, except that it encodes for production of colicins B and M rather than ColV.

At least portions of these plasmid-linked PAIs appear to occur widely among APEC isolated from different parts of the world (9, 102, 103, 132, 243, 329, 335, 435, 436, 523, 553, 561), various avian host species (9, 335, 435, 436), and different syndromes (98, 436). These observations suggest that the conserved region of these plasmid-linked PAIs might be a defining characteristic of the APEC pathotype (436) that could be exploited in colibacillosis control. Protocols for rapid characterization of APEC, based on detection of certain virulence genes, including some from this cluster, show promise (133, 474).

Regulation of APEC Virulence

Mutational analysis of the specific phosphate transport system (*pst*) operon of an APEC strain resulted in deregulation of phosphate sensing and changes in the composition of the bacterial surface. These changes were accompanied by increased susceptibility to serum, acid shock, and polymyxin, and resulted in decreased virulence, suggesting that a functional Pst system is required for full virulence of APEC O78 strain χ7122 (297). Also, the BarA-UvrY two-component system has been shown to regulate APEC virulence. Mutants lacking *barA* or *uvrY* had impaired adherence, invasiveness, persistence in tissues, survival in macrophages, and serum resistance (211). It is likely that completion of the APEC genome will facilitate experimentation that will provide insight into the critical issue of virulence regulation.

Pathobiology and Epidemiology

Incidence and Distribution

Escherichia coli have a cosmopolitan distribution. The various serotypes of *E. coli* are normal intestinal inhabitants and occur in high numbers in most animals including human beings. Presence of *E. coli* in the lower intestinal tract is beneficial, aiding in growth and development (460) and inhibiting other bacteria including *Salmonella* (148, 331, 419). *E. coli* likely occur in most mammals and birds although healthy psittacines may be an exception (25, 493). It is a common inhabitant in the intestinal tracts of poultry at concentrations up to 10^6 *E. coli*/gram of intestinal contents. Higher numbers are found in younger birds, birds without an established normal flora, and in the lower intestinal tract (113, 304, 546). A diversity of *E. coli* types colonize the cecal mucosa, which may shift abruptly as birds age (247). *E. coli* strains that persist over time are considered resident strains while those that only are present for a limited period are considered transient strains. Among normal chickens, 10–15% of intestinal coliforms may belong to potentially pathogenic serotypes (204) although intestinal strains may not be the same serotype as those from extra-intestinal sites in the same bird. Intestinal *E. coli* provide a reservoir for virulence and antimicrobial resistance factors (391).

Egg transmission of pathogenic *E. coli* is common and can be responsible for high chick mortality (163, 312, 412, 440). Fluoroquinolone-resistant *E. coli* were vertically transmitted from clinically normal breeders and caused high mortality in chicks (412). Pathogenic coliforms are more frequent in the intestine of newly hatched chicks than in the eggs from which they hatched (203), suggesting rapid spread after hatching. The most important source of egg infection seems to be fecal contamination of the egg surface with subsequent penetration of the shell and membranes.

Coliform bacteria can be found in litter and fecal matter. However, *E. coli* accounts for only a small number of total bacteria in litter (382). Environmental isolates constituted a distinctly different population from APEC occurring in the flock (244). Dust in poultry houses may contain 10^5–10^6 *E. coli*/g. Dust concentrations are higher immediately outside of power-ventilated houses than they are within the houses. *E. coli* are present in the air inside of the houses and up to 40 ft outside of the houses (96). These bacteria persist for long periods, particularly under dry conditions (202). Wetting dust inside houses with water resulted in an 84–97% reduction within 7 days. Feed and feed ingredients are often contaminated with pathogenic coliforms and are a common source for introducing new serotypes into a flock (324). Rodent droppings frequently contain pathogenic coliforms. The intestinal tract of the mouse is a suitable environment for transfer of genes from resistant to susceptible strains. Exposure of mice to an antibiotic accelerates the process (205). Pathogenic serotypes also can be introduced into poultry flocks through contaminated well water (368). Presence of

E. coli in drinking water indicates fecal contamination and can serve as an indicator for the potential presence of any fecal-oral transmission of infectious agent.

Natural and Experimental Hosts

Most, if not all, avian species are susceptible to colibacillosis. Clinical disease is reported most often in chickens, turkeys, and ducks. Collectively, the various forms of colibacillosis are considered to be the most common infectious bacterial disease of broiler chickens and turkeys. Natural infections of quail (16, 64, 141, 562), pheasant (500), pigeons (56, 429), guinea fowl (199, 315), waterfowl (33, 50, 88, 239, 340, 426, 531), ostriches (85, 274, 385, 538), and emus (214, 545) have been reported.

Age of Host Commonly Affected

All ages are susceptible to colibacillosis, but young birds are more frequently affected and severity of disease is greater in young birds, including developing embryos (175, 200, 253, 348). Outbreaks can occur in caged layers (523, 560) and coliform salpingitis/peritonitis is a common cause of mortality in breeders (260).

Host Susceptibility Factors

Compared with bacterial virulence factors, host susceptibility and resistance factors are probably an equal or greater determinant of colibacillosis occurrence (Tables 18.4, 18.5). Normal, healthy birds with intact defenses are remarkably resistant to naturally occurring *E. coli* exposure including virulent strains. Infection occurs when skin or mucosal barriers are compromised (e.g., unhealed navel, wounds, mucosal damage from viral, bacterial, or parasitic infections, lack of normal flora, etc.), the mononuclear-phagocytic system is impaired (e.g., viral infections, toxins, nutritional deficiencies), there is immunosuppression (e.g., viral infections, toxins), exposure is overwhelming (e.g., environmental contamination, poor ventilation, contaminated water), and/or birds are exposed to abnormal stress or have an inappropriate response to stress. Effective control of colibacillosis depends on identifying and eliminating the predisposing cause(s) of the disease.

Colibacillosis often occurs concurrently with other diseases making it difficult to determine the contribution of each agent to the overall clinical disease. For example, colibacillosis, paraty-

Table 18.4. Factors known or suspected to increase host susceptibility to *Escherichia coli* infections in poultry. See also 28.

Factor	References	Factor	References
Viruses		**Toxins**	
Adenovirus (Type 1)	(106, 107, 214, 443, 530)	Ammonia	(367, 397)
Avian pneumovirus	(7, 246, 322, 514, 516)	Cyclophosphamide	(105, 123, 373)
Chicken infectious anemia virus	(427)	Iron—parenteral	(57)
Duck enteritis virus (low virulent)	(467)	Mycotoxins	
Hemorrhagic enteritis virus	(387, 416, 518)	Ochratoxin	(283, 284)
Infectious bronchitis virus	(83, 161, 327, 328, 378, 478)	Fumonison/Moniliformin	(309, 310)
Infectious bursal disease virus	(375, 383, 443)	**Physiologic**	
Infectious laryngotracheitis virus	(313, 378)	Age—young	(175, 253, 348)
Influenza virus	(26, 278, 389, 502)	Stress-minimal or severe	(224, 326)
Marek's disease virus	(143)	Sex—male	(220)
Newcastle disease virus	(127, 128, 184, 377, 415, 416)	Fast-growing strains	(557)
Pigeon paramyxovirus 1	(551)	Obesity	(395)
Reovirus	(443)	High antibody response	(188)
Turkey coronavirus	(192, 384)	High inflammatory response	(37)
Pigeon circovirus	(429, 444)	**Environmental**	
"Stunting Syndrome"	(144)	Contaminated water	(369)
Bacteria		Dry, dusty conditions	(202)
Bordetella avium	(138, 215, 416, 515)	Feed/water restriction	
Pasteurella multocida	(483)	Inadequate ventilation	
Campylobacter jejuni	(166)	Overcrowding	
Clostridium perfringens (?)	(358)	Poor litter conditions	
Mycoplasma gallisepticum	(184, 377)	Temperature extremes	
M. meleagridis	(350, 389, 415, 452)	**Nutrition**	
M. synoviae	(320, 486, 498)	Hypervitaminosis E	(145)
Chlamydiophila psittaci	(519)	Hypervitaminosis A	(142)
Parasites		Vitamin A deficiency	(142)
Ascaridia (larvae)		**Other**	
A. dissimilis	(394)	Trauma (bruising)	(195)
A. galli	(411)		
Eimeria brunetti	(207, 368)		
Eimeria tenella	(374)		
E. tenella/whole wheat diet	(150)		
Cryptosporidium baileyi			
Histomonas meleagridis	(60, 333, 485)		

phoid, and histomoniasis caused high mortality in a broiler flock maintained free of antibiotics where high ambient temperatures and humidity may have been additional factors contributing to the disease (151). High mortality in Japanese quail with signs of respiratory disease was associated with *Mycoplasma gallisepticum*, *Pasteurella multocida*, *Staphylococcus* sp., *Streptococcus* sp., *Cryptosporidium* sp., and *E. coli*; relatively high ammonia levels likely also contributed to the clinical disease (359).

Infection with infectious bronchitis virus (IBV) in chickens (83, 161, 378, 478), infection with hemorrhagic enteritis virus in turkeys (387, 416, 518), and exposure of avian species to ammonia (367, 397) are the most commonly reported factors that predispose to colibacillosis. Interactions between IBV and *E. coli* have been studied extensively and used to determine virulence of both organisms, efficacy of IBV vaccination programs, and effect of IBV vaccination on subsequent colibacillosis (84, 327, 328, 376). IBV vaccination of chicks at one day of age by spray reduced the occurrence and severity of airsacculitis following challenge with virulent IBV and *E. coli.* In contrast IBV vaccination by eye-drop reduced systemic infection and improved uniformity, but did not protect against airsacculitis (328).

Moderate stress increases resistance, possibly as a result of the development of immunity following contact of organisms with the immune system (304), or as a result of developing and exercising defense mechanisms and maintaining them in a state of readiness (185). Similarly, provoking mild, nonspecific inflammation of the respiratory system increases resistance to subsequent respiratory *E. coli* infection (511). Resistance to intra-air sac *E. coli* challenge was observed following primary NDV vaccination with the Roakin strain, which was prevented when the chickens were given corticosterone (217). Individual survival is likely promoted by diversion of feed-derived nutrients from growth and development to antibacterial defenses (187). Protein does not accumulate at the same rate in muscles of infected birds once they recover and they do not match the weight-for-age of uninfected birds (509). Inhibition of prostaglandin E2 by naproxen restored normal growth (508), which is consistent with the earlier finding that inhibition of prostaglandins with aspirin and vitamin E decreased the severity of disease resulting from *E. coli* challenge (311).

Genetic lines of chickens and turkeys vary in their resistance to *E. coli* infections (21, 469, 470, 557). Variations among genotypes in growth rate, nutritional interactions, and immune responsiveness that relate to *E. coli* susceptibility also have been identified. Consistent among studies on both chickens and turkeys is an inverse relationship between growth rate and resistance to colibacillosis (188, 218, 227, 470, 557, 558). Selection for rapid growth is believed to require redirection of nutrients towards growth at the expense of bacterial resistance (187, 428). However, no correlation between body weight at market for broilers or chick production of breeders with high early antibody response to *E. coli* vaccine was found, indicating the feasibility to select for both immune responsiveness and desirable production traits (305). Immune responses to other vaccines and antigens paralleled response to *E. coli* vaccine in selected lines (559). In general, chickens and turkeys that are more immunologically responsive (e.g., high early antibody lines) are more susceptible to colibacillosis unless they have been vaccinated or otherwise exposed prior to challenge (37, 120, 558). Genetic lines may be more resistant to colibacillosis if the challenge method uses a predisposing agent such as infectious bronchitis virus because of resistance to the predisposing agent rather than *E. coli* (63). When 5 broiler lines, a slow-growing line, and 2 line crosses were examined using a standardized pure *E. coli* challenge, substantial differences in mortality, lesion occurrence, and growth depression were found. These results indicated that selection for resistance would be feasible, but that heterosis was either negative or negligible making test crossings essential (21, 22). Evaluation of 4 broiler strains for their response to endotoxin revealed differences in weight gain and changes in bone breaking strength. Response of the strains to endotoxin with regard to changes in liver size and bone breaking strength were highly correlated with mortality prior to endotoxin exposure. Strains that had a greater loss of bone breaking strength as a result of inflammation were more likely to have higher overall mortality (227, 341). Variations in physiological and behavioral responses to endotoxin also occur among egg-laying strains (74).

Table 18.5. Factors known or suspected to decrease host susceptibility to *Escherichia coli* infections in poultry. See also 28.

Factor	**Nutrition**
Immunity	Protein
Passive	Vitamin A
Active	Vitamin C
Immunostimulants	Vitamin D
Phagocyte priming	Vitamin E
Physiologic	ß-carotene
Genetics	High iron—oral
Age—older	Selenium
Sex—Female	
Moderate stress	
Socialization	
Deoxycorticosterone	
Short heat stress	
Intestinal flora	

Transmission, Carriers, Vectors

Escherichia coli is present in the intestinal tracts of most animals and shed in the feces, often in high numbers. Direct or indirect contact with other animals or feces can introduce new strains into the poultry flock. Free-living birds are especially important as they are colonized with strains that are already adapted to avian species. *E. coli* is readily isolated from free-living waterfowl, especially ducks (82, 135, 136), and passerine birds, especially European starlings (355). A particularly virulent O86 APEC has caused significant mortality in free-living finches in Britain, but has yet to be found in poultry (140, 410).

Trachea, ceca, and oviduct of recovered laying hens remained persistently colonized for at least 21 weeks after either oral or intra-air sac inoculation with pathogenic *E. coli.* Hens with colonized oviducts continued to lay eggs of which 2.7% contained the

organism. Interestingly, *E. coli* was not isolated from the shell surface, even when the oviduct was heavily colonized (15).

Larval and adult darkling beetles (*Alphitobius diaperinus*) likely contribute to *E. coli* transmission and its spread among poultry houses and farms following consumption of infected larvae or beetles or contact with their feces by the birds (171). Following exposure, larvae and adults were positive for *E. coli* both externally and internally for up to 12 days. The organism was shed in their feces for 6–10 days. Chicks became colonized with *E. coli* after eating infected larvae or adults, but the number of infected chicks was higher when the birds ate larvae (332).

Adult houseflies (*Musca domestica*) serve as mechanical vectors of *E. coli,* and fly larvae develop digestive tract infections with *E. coli* following ingestion of bacteria-laden material. Ingested bacteria can serve as a food source for flies; larvae have a high survival rate when experimentally fed solely *E. coli*. Once infected, *E. coli* persists through the pupal and adult stages making it possible for flies to serve as a reservoir for virulent strains (432, 433). The gut of the housefly provides a suitable environment for horizontal transfer of *E. coli* antibiotic resistance and virulence genes (413).

Incubation Period

The time between infection and onset of clinical signs varies with the specific type of disease produced by *E. coli.* The incubation period is short, generally between 1 and 3 days, in experimental studies in which birds are exposed to high numbers of virulent organisms. In the field it is more common to see colisepticemia 5–7 days after infection with a predisposing agent such as infectious bronchitis virus in chickens or hemorrhagic enteritis virus in turkeys.

Clinical Signs

Clinical signs vary from inapparent to total unresponsiveness just prior to death depending on the specific type of disease produced by *E. coli.* Localized infections generally result in fewer and milder clinical signs than systemic diseases. Coliform cellulitis is typically not detected until the birds are processed. Lameness and retarded growth are seen in birds with skeletal lesions that develop as a sequel to sepsis. Affected birds are typically undersized for the flock and found at the ends of the house, along the side walls, or under feeders or waterers. They may be victims of persecution ("cannibalism") by other birds. When joints or bones of one leg are affected, birds walk with a characteristic hopping motion to keep weight off of the affected leg. Birds with lesions in both legs are either nonambulatory or have great difficulty in standing and walking. When the thoracolumbar spine is affected, the birds have an arched back, sit on their hocks, and bear little or no weight on their feet. Occasionally they will sit back on their tail and hocks with their feet elevated off the ground. Birds with chronic lameness have caking of droppings around the vent and on abdominal feathers. Feces are green with white to yellow urates as a result of anorexia and dehydration. Young birds with omphalitis and infected yolk sacs also may have difficulty in walking because of abdominal distention, which alters weight distribution and impairs balance.

Birds with colisepticemia are often terminally moribund and the flock may be inactive and not eating. Decreased water consumption is associated with a poor prognosis. Severely affected individual birds are unresponsive when approached, do not react to stimuli, and are easily caught and handled. They sit with their eyes closed in a hunched position with drooping of the head, neck, and wings. The beak may be inserted into the litter to support the head. Dehydration is indicated by dark dry skin, which is especially noticeable in the shanks and feet. Dehydrated young chicks typically have prominent raised folds of skin along the medial and lateral sides of the shanks and toenails that appear black. Although, technically, death is not a clinical sign, this may be the main indication of an outbreak of colibacillosis in a flock.

Clinical signs of predisposing or compounding factors often are seen concurrently with signs of *E. coli* infections.

Morbidity and Mortality

Both morbidity and mortality are highly variable depending on the type of disease produced by *E. coli*. It is probable that most, if not all, commercial flocks experience some degree of morbidity, mortality, or condemnation due to *E. coli* infections.

Mortality occurring during the day indicates a more severe disease outbreak than when birds are only found dead in the morning. In flocks with highly virulent colisepticemia it is occasionally possible to watch a bird sicken and die within a few hours. A flock that appears clinically normal when examined during the day, but has an excess number of dead birds the following morning, is a common finding in mildly affected flocks. This pattern is typical for egg layer and breeder flocks experiencing coliform salpingitis/peritonitis.

Pathology

Several localized and systemic types of colibacillosis affect poultry. Often the name is based on the tissue(s) affected or disease process (Table 18.6).

Localized Forms of Colibacillosis

Coliform Omphalitis/Yolk Sac Infection. Omphalitis is an inflammation of the navel (umbilicus). In birds the yolk sac is usually involved too because of its close anatomic relationship. Infection follows contamination of the unhealed navel with APEC. Fecal contamination of eggs is considered to be the most important source of infection. Bacteria may be acquired *in ovo* if the hen has oophoritis or salpingitis or via contamination following artificial insemination (201, 348). Yolk sac infections also can result from translocation of bacteria from the chick's intestine or from the bloodstream. In these cases the navel is not affected.

It is common to recover low numbers of *E. coli* from normal yolk sacs. Between 0.5 and 6% of eggs from normal hens contain *E. coli.* Experimentally inoculated hens may shed *E. coli* in up to 26% of their eggs. Pathogenic strains accounted for 43 of 245 isolates from dead embryos (200). About 70% of chicks with "mushy chick disease" had *E. coli* in their yolk sacs (200). Other types of bacteria also can cause omphalitis, although *E. coli* is most common.

Adhesin factors characterizing omphalitis isolates of *E. coli*

Table 18.6. Classification of the different types of pathological manifestations of colibacillosis (adapted from (29)).

Manifestation
Localized Infections
Coliform Omphalitis/Yolk Sac Infection
Coliform Cellulitis (inflammatory process)
Swollen Head Syndrome
Diarrheal Disease
Venereal Colibacillosis (acute vaginitis)
Coliform Salpingitis/Peritonitis/Salpoperitonitis (adult)
Coliform Orchitis/Epididymitis/Epididymo-orchitis
Systemic Infections
Colisepticemia
Respiratory-Origin (Air Sac Disease, Chronic Respiratory Disease, CRD)
Enteric-Origin
Neonatal
Layer
Duck
Colisepticemia Sequelae
Meningitis/Encephalitis
Panophthalmitis
Osteomyelitis
Spondylitis
Arthritis/Polyarthritis
Synovitis/Tenosynovitis
Sternal Bursitis
Chronic Fibrosing Pericarditis
Salpingitis (Juvenile)
Coligranuloma

included type 1 (F) fimbriae in 96%, P fimbriae in 8%, and afimbrial adhesin in 16%. Afimbrial adhesin occurred more frequently in omphalitis isolates compared to isolates from cases of salpingitis, swollen head syndrome, or respiratory disease (275). When genotyped, omphalitis isolates tended to be more similar to commensal isolates than they were to isolates from swollen head syndrome or septicemia (11, 93). A high percentage of *E. coli* isolates from eggs, dead embryos, and chicks that died between placement and 7 days of age possessed the virulence genes *ipaH* (invasion and persistence in cells), *eae* (attaching and effacing lesions), and *cdt* (cell distension and death) compared to other APEC (440).

Some embryos may die before hatching, particularly late in incubation; whereas others die at or shortly after hatching. Surviving infected chicks can be a source of *E. coli* for other chicks in the same hatch (348). The incidence of birds with omphalitis increases after hatching and declines after about 6 days with occasional losses continuing up to 3 weeks. As few as 10 organisms of serotype O1a:K1:H7 caused 100% mortality in day-old chicks following yolk sac injection (468). When birds become infected with low virulent strains there may be no embryo or chick mortality or some may survive although hatchability, chick livability, and relative yolk weights may be affected (348); the only pathologic finding being retention of infected yolk sacs containing caseated yolk (181).

Swelling, edema, redness, and possibly small abscesses characterize acute inflammation of the navel. The abdomen is distended and blood vessels are hyperemic (Figure 18.1B). In severe cases the body wall and overlying skin undergo lysis and are wet and dirty. These birds are referred to as "mushy" chicks or poults. There may be other nonspecific changes such as dehydration, visceral gout, emaciation, vent pasting, and enlarged gall bladder. The yolk sac is typically distended because yolk has not been absorbed and inflammatory products have been added. Yolk is abnormal in color, consistency, and smell, and may contain visible exudate. Blood vessels of the yolk sac are often prominent (Figure 18.1A). Chicks or poults with infected yolk sacs that live more than 4 days also may have pericarditis or perihepatitis, indicating systemic spread of the organism from the yolk sac.

Microscopically the wall of the infected yolk sac is edematous with mild inflammation. There is an outer connective tissue zone adjacent to a layer of inflammatory cells containing heterophils and macrophages, a layer of giant cells, a zone of necrotic heterophils and masses of bacteria, and then the inner, abnormal yolk contents. A few plasma cells may be found in some yolk sacs.

Omphalitis and yolk sac infection have been experimentally reproduced in ducks by exposing eggs to *E. coli* broth cultures. Dipping eggs at 18 days of incubation resulted in a higher incidence of infection than dipping at 1 day. Low brooding temperature or fasting after hatching increased the incidence of infection and mortality (459).

Consequences of yolk sac infection include deprivation of nutrients and maternal antibodies, absorption of toxins, and spread of *E. coli* by extension into the body cavity or systemically to produce colisepticemia. Survivors are usually stunted and do poorly. Subsequently the yolk sac contracts but an abscess remains for an extended period. *E. coli* often persists in the inflamed yolk sac for weeks or months. Adhesions to intestines, especially the tip of the duodenal loop, or other visceral organs are common. Rarely the elongated stalk of the yolk sac will wind around the intestine and cause strangulation.

Coliform Cellulitis (Inflammatory Process). Cellulitis is an inflammation of the subcutis that extends beneath normal skin. Cellulitis is rare in mammals but relatively common in birds. It may have many causes, but *E. coli* infection is most common in chickens. For this reason, the term cellulitis has been used synonymously with coliform cellulitis. However, cellulitis in turkeys is not frequently associated with *E. coli* infection (65, 168, 396). Cellulitis is covered separately below because this form of colibacillosis has emerged as a significant disease problem in broiler chickens.

Swollen Head Syndrome. Swollen head syndrome (SHS) is an acute to subacute cellulitis involving the periorbital and adjacent subcutaneous tissues of the head (Fig. 18.2G). SHS was first described in broilers in South Africa associated with *E. coli* and an unidentified coronavirus infection (356). The disease has subsequently been described in most intense poultry-producing areas of the world. The disease also affects turkeys and guinea fowl (315, 516).

Swelling of the head is caused by inflammatory exudate beneath the skin that accumulates in response to bacteria, usually *E. coli,* following upper respiratory viral infections (e.g. avian pneumovirus, infectious bronchitis virus). Ammonia aggravates the disease (118). The portal of entry is considered to be the conjunctiva or inflamed mucous membranes of the sinuses or nasal cavity (379). Possible infection via the eustachian tube also has been suggested (118). Microscopic lesions include fibrinoheterophilic inflammation and heterophilic granulomas in the air spaces of the cranial bones, middle ear, and facial skin. Lymphoplasmacytic conjunctivitis and tracheitis with formation of germinal centers have also been observed (238).

Although the pathogenesis of SHS has not been established, conjunctival-associated lymphoid tissue inflamed from virus infection and/or ammonia irritation may serve as the site through which bacteria gain access to subcutaneous tissues. Periorbital inflammation is typically seen early in the disease and hyperplastic lymphoid tissue has been shown to be a site where *E. coli* penetrates mucosal surfaces (184). Scarifying the conjunctival mucosa and instilling a pure culture of *E. coli* (356), or inoculation of *E coli* into submucosal or subcutaneous tissues (380), will reproduce the disease. Intranasal inoculation of avian pneumovirus and *E. coli* failed to reproduce the disease (380). SHS did not occur when day-old chicks were inoculated supraconjunctivally with either avian pneumovirus or *E. coli*, but they did develop clinical disease, which was most severe when the chicks received both agents (7).

Escherichia coli isolates from SHS cases possessed several virulence factors including fimbrial adhesins, colicin production, aerobactin, and complement resistance. In general SHS isolates had virulence attributes that were similar to isolates from cases of septicemia (93), except colicin production and iron-acquisition siderophores were more frequent in SHS isolates. The colicins produced by SHS strains often differed from ColV (92). The majority of strains were motile, but presence of K1 capsule was infrequent (401). Similar results were found in a subsequent study in which SHS isolates were more similar to isolates that cause septicemia than isolates that cause omphalitis or commensal isolates except type 1 and curli fimbriae, and temperature-sensitive hemagglutinin (tsh) were more frequent in SHS isolates (11). A transferable 60 MDa plasmid from an SHS *E. coli* isolate contained genes for cell adhesion, colicin production, and tsh (489). Adhesin factors occurring in SHS *E. coli* isolates included type 1 in 94% and P fimbriae in 28%. The P-fimbrial adhesin factor occurred more frequently in SHS isolates compared to isolates from cases of salpingitis, omphalitis, or respiratory disease (275). A unique Shiga toxin (VT2y) (454) that may be involved in the pathogenesis of SHS was identified in a high percentage of SHS *E. coli* isolates (402). Additionally another toxin, similar to one produced by *Bacillus cereus* that is highly lethal for mice following injection, was identified in SHS isolates (456).

Diarrheal Disease. Primary enteritis is a common manifestation of *E. coli* infections in mammals including human beings, but is considered rare in poultry. Diarrhea results from infections with enterotoxigenic (ETEC), enterohemorrhagic (EHEC), enteropathogenic (EPEC), or enteroinvasive *E. coli* (EIEC); each type possessing certain virulence factors that determine the characteristics for each type of enteric disease (see Virulence Factors). EHEC and EPEC strains produce attaching and effacing lesions on intestinal mucosal surfaces. Collectively, these strains are called attaching and effacing *E. coli* (AEEC). Intestinal *E. coli* in poultry have been poorly studied, except as commensal strains for comparison with APEC or reservoirs of virulence genes that occur in human strains, so our knowledge of the role that *E. coli* may play in intestinal disease is limited.

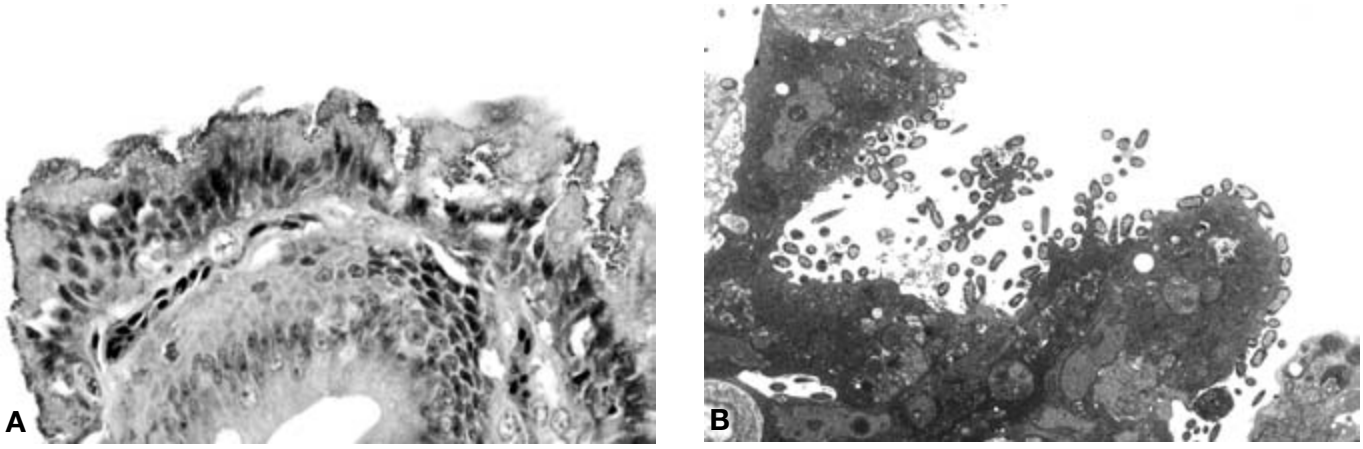
Enterotoxigenicity caused by ETEC strains is uncommon in APEC. Most surveys for heat stable and heat-labile enterotoxins either fail to find any positive isolates or identify only a few (130). ETEC that elaborated toxins capable of causing fluid accumulation in ligated intestinal loops of chickens were recovered from chickens with diarrhea (5, 261), and an O15 APEC strain that produced heat-labile toxin II was isolated from ostrich chicks experiencing severe diarrhea and high mortality (385).

Natural and experimental infections with AEEC or presence of *eae* gene have been reported in chickens (149, 264, 281, 440, 492, 495), turkeys (398, 399, 492), pigeons (530), ducks (492), psittacines (463), and other avian species (140, 293). Infections with infectious bursal disease virus in chickens and adenovirus infection in the pigeon were considered possible predisposing factors to AEEC infection. In turkey poults, coinfection of EPEC and turkey coronavirus (TCV) resulted in severe stunting and very high mortality (192). Clinical disease was most severe when poults were infected with TCV prior to inoculation with EPEC (399). Ten of 12 commercial turkey flocks experiencing high mortality because of poult enteritis mortality syndrome (PEMS) were infected with EPEC confirming the importance of natural EPEC infection as a cause of mortality in young turkeys (399).

Birds infected with AEEC may be clinically normal or have diarrhea and be dehydrated. In clinically affected birds, the intestines are pale and distended with fluid, which may contain visible flecks of mucus and exudate. Ceca are often the most obviously affected part of the digestive tract. They are typically distended with pale brown fluid and gas. Bacteria intimately attach to the surface of enterocytes causing effacement of microvilli, pitting, and pedestal formation, which are best seen by electron microscopy (Figure 18.3). Lesions are most common in the ceca. Organisms are readily identified in tissue sections using Giemsa stain or by immunohistochemical methods.

Attempts to experimentally define a role for *E. coli* in malabsorption syndrome of chickens were not successful (347, 481, 482). In contrast, specific strains of *E. coli* have been associated with PEMS (123, 124). Turkey astrovirus, an agent involved in PEMS, impairs macrophage function, which could explain the enhanced susceptibility of affected poults to secondary bacterial infections such as colibacillosis (425).

Diseases resulting from infection with EIEC have not been described but are likely, especially in the case of neonatal septicemia. EIEC possess genes such as *ipa,* which encodes a virulence factor that provides the organism with the ability to penetrate and survive within cells. The most frequently identified virulence gene in *E. coli* isolated from eggs, dead embryos, and



18.3. Attaching effacing *E. coli* bind tightly to the apical surface of enterocytes destroying the normal brush border. On light microscopy the surface epithelium appears irregular, and numerous bacteria can be seen attached to affected cells (A). By EM the organisms characteristically occupy small pits in the cell surface or are on pedestals. The number of bacteria and extent of brush border effacement can be clearly seen (B). (Courtesy of Dr. H.L. Shivaprasad)

chicks with omphalitis/yolk sac infections was *ipaH*. Most of the *ipaH*$^+$ isolates (62/80; 77.5%) came from liver or yolk sac of chicks that died between 3 and 7 days of age, which corresponded to a period of increased mortality (440). Further characterization of the *ipaH*$^+$ isolates revealed properties that did not match those of typical EIEC and the existence of specific EIEC clone complexes among avian isolates. Cell invasion was confirmed *in vitro* (440).

Venereal Colibacillosis (Acute Vaginitis). Venereal colibacillosis is an acute and frequently fatal vaginitis that affects turkey breeder hens shortly after they are first inseminated. Puncturing the hymen of young turkey hens can lead to a severe localized *E. coli* infection characterized by vaginitis, cloacal and intestinal prolapse, peritonitis, egg binding, and internal laying. The affected mucosa is markedly thickened, ulcerated, and covered with a diphtheritic, caseo-necrotic membrane, which causes obstruction of the lower reproductive tract. The marked tissue changes can lead to egg binding. The upper oviduct is grossly and histologically normal. Flocks can have losses of up to 8% because of increased mortality and culling. Egg production is decreased and there is an increased number of cull eggs due to small size. No other infectious agents have been identified as contributing to the disease (153).

Coliform Salpingitis/Peritonitis/Salpingoperitonitis (Adult). Inflammation of the oviduct caused by *E. coli* results in decreased egg production and sporadic mortality. It is one of the most common causes of mortality in commercial layer and breeder chickens (48, 49, 260) and also affects other female birds, especially ducks and geese (50). Accumulations of caseating exudate in the body cavity resemble coagulated yolk, which is the reason for the common name "egg peritonitis." Salpingitis and egg binding may occur concurrently. They are often confused with each other because they both result in an obstructive mass within the oviduct. Yolk peritonitis is a mild to moderate diffuse peritonitis that results from free yolk in the body cavity. Marked exudation, extensive inflammation, and positive cultures characterize coliform peritonitis and serve to distinguish it from yolk peritonitis.

Infection occurs when *E. coli* ascends the oviduct from the cloaca. Injecting large (10^9) numbers of bacteria into the reproductive tract will reproduce the disease. Mucosal infections with viruses (*e.g.* infectious bronchitis virus) or mycoplasmas also may predispose a bird to salpingitis. Co-infection with *E. coli* and *Tetratrichomonas* occurred in Pekin duck breeders with salpingitis (88). Heavy egg production and associated estrogenic activity predispose hens to salpingitis by relaxing the sphincter between the vagina and cloaca. Spread to the oviduct from an airsacculitis is also possible but this form of salpingitis occurs more frequently in young birds as part of a systemic infection.

Isolates of APEC from birds with salpingitis have similar virulence characteristics to those that cause airsacculitis. In a study of 30 isolates, 11 belonged to serogroups O2 and O78 while 10 were untypeable. Twenty-seven of the isolates were of either high or intermediate virulence in a day-old chick assay. Most isolates possessed type 1 fimbriae and adhered to oviduct epithelium, especially in adult breeders, and they had the ability to acquire iron when grown in iron-deficient medium. Isolates were resistant to serum from young breeders, but sensitive to serum from older breeders (346). In a separate study type 1 fimbriae also were identified in 49 of 50 isolates from broiler breeders with salpingitis; few isolates possessed other fimbrial types (275). Therefore presence of type 1 fimbriae can be considered characteristic of APEC salpingitis isolates.

In chronic cases the oviduct is markedly distended and thin-walled with single or multiple masses of caseous exudate in the general form of the oviduct (Figure 18.2A). The mass of exudate may expand to the point that it fills most of the body cavity (97). Exudate is laminated, often contains a central egg, shells, and/or membranes, and is malodorous. Spread of the organism into the body cavity through the compromised oviduct wall leads to con-

current peritonitis, which is termed salpingoperitonitis when there is involvement of both the oviduct and peritoneum. Peritonitis in the absence of salpingitis also can occur, but is uncommon. Acute cases have less exudate in the oviduct or peritoneum that tends to be soft and not as caseated (Figure 18.2B). Affected birds are incapable of producing and laying eggs. Abdominal laying and misovulated ova may accompany salpingitis and contribute to peritonitis.

Microscopically the tissue reaction in the oviduct is surprisingly mild in view of the marked gross lesions. It primarily consists of multifocal to diffuse heterophil accumulations subjacent to the epithelium and caseous exudate in the lumen, which often contains bacterial colonies. Lymphoid foci develop in the mucosa with time and indicate chronicity.

Coliform Orchitis/Epididymitis/Epididymo-orchitis. An ascending *E. coli* infection of the male reproductive tract, analogous to that resulting in salpingitis in the hen, occurs infrequently in roosters. Testicles are swollen, firm, inflamed, irregularly shaped, and have a mosaic of necrotic and viable tissue when opened. Heavy growth of *E. coli* can be obtained from the testicle and epididymis (345).

Systemic Forms of Colibacillosis

Colisepticemia. Presence of virulent *E. coli* in the blood stream defines colisepticemia. Virulence and number of organisms balanced against efficacy of host defenses determine duration, degree, and outcome of the disease, as well as the pattern and severity of lesions (420, 421). Colisepticemia progresses through the following stages: acute septicemia, subacute polyserositis, and chronic granulomatous inflammation (75). While lesions are typical of colisepticemia, other bacteria capable of producing septicemia also can cause similar changes. Characteristic features of colisepticemia at necropsy are tissues that develop a green discoloration following exposure to air and a characteristic odor, possibly related to indole produced by the organism. The bursa of Fabricius is often atrophic or inflamed as a result of colisepticemia. It should not be interpreted that a small bursa is evidence of a prior immune suppressing disease such as infectious bursal disease (114, 370, 371).

Pericarditis is common and is a characteristic of colisepticemia. It is usually associated with myocarditis, which results in marked changes in the electrocardiogram (182), often before gross lesions appear. Vessels in the pericardium become increasingly prominent because of hyperemia and the pericardium becomes cloudy and edematous. Initially fluid and soft masses of pale exudate accumulate within the pericardial sac followed by fibrinous exudate (Fig. 18.1C). Exudate can be seen loosely adhering to the epicardium when the pericardial sac is opened. As the disease progresses exudate increases, becomes more cellular (fibrinoheterophilic), and undergoes caseation. The pericardial sac adheres to the epicardium with chronicity.

Microscopically the same progression of lesion development is seen. Serous and serofibrinous exudate is seen initially followed by increasing numbers of heterophils and subsequently macrophages. Within the myocardium, particularly close to the epicardium, there are accumulations of lymphoid cells and by 7–10 days there also are many plasma cells. Subsequently, exudate in the pericardial sac undergoes organization (Fig. 18.1G), which, in survivors, eventually results in constrictive pericarditis and liver fibrosis due to chronic passive congestion. Cardiac lesions reduce arterial blood pressure from a norm of about 150 mmHg to about 40 mmHg just before death.

Several distinct clinical forms of colisepticemia can be distinguished depending on how the organism gains access to the circulation and the age and type of bird.

Respiratory-Origin Colisepticemia. Respiratory-origin colisepticemia affects both chickens and turkeys and is the most common type of colisepticemia. *E. coli* gains access to the circulation following damage to the respiratory mucosa by infectious or noninfectious agents (161, 180). Infectious bronchitis virus (IBV) and Newcastle disease virus (NDV), including vaccine strains, mycoplasmas, and ammonia, are the most common predisposing agents. Avian pneumovirus infection increases susceptibility of turkeys to colisepticemia (7, 246, 514, 516). Severity of the resulting disease, which is commonly referred to as air sac disease, chronic respiratory disease (CRD), or multicausal respiratory disease (215), is directly related to the number of agents that are involved. A diversity of *E. coli* serotypes can be identified in a disease outbreak. Those in the tissues are usually different from those in the intestinal tract of the same bird but can be found in the intestinal tracts of other birds and the environment.

Susceptibility is increased when only IBV or NDV infection occurs. Five days after administration of a vaccine strain of NDV, clearance of aerosol-administered *E. coli* is reduced. Microscopically 3–8 layers of immature, nonciliated cells replace the pseudostratified, columnar epithelium of the trachea (139). Mixed IBV-*E. coli* infections are more severe than those caused by either agent alone (378, 478). Antibodies against *E. coli* produced by chickens infected with infectious bursal disease virus and/or IBV had a significant decrease in opsonizing ability compared to antibodies produced by normal chickens. Reduced opsonization of the organism resulted in decreased macrophage function, which may help explain the frequent infection with *E. coli* that follows IBV infection (383).

Mycoplasmal infection increases susceptibility to *E. coli* about 12–16 days postinoculation, and susceptibility persists for at least 30 days. Infection with IBV or NDV in addition to mycoplasma further decreases resistance to *E. coli* and the period of increased susceptibility begins earlier and persists longer.

Inhaled coliform contaminated dust has been implicated as one of the most important sources for infecting air sacs of susceptible birds. Exposure to chicken-house dust and ammonia results in deciliation of the upper respiratory tract of birds (367, 397) permitting inhaled *E. coli* to colonize and cause respiratory infection.

Lesions are prominent in respiratory tissues (trachea, lungs, air sacs), pericardial sac, and peritoneal cavities and are typical of the subacute polyserositis stage of colibacillosis (75, 421) (Figs. 18.1C–F). Infected air sacs are thickened and often have caseous

exudate on the respiratory surface. Microscopically the earliest changes consist of edema and heterophil infiltration. Mononuclear phagocytes are frequently seen 12 hours after inoculation. Later, macrophages become common, with giant cells along margins of necrotic areas. There is fibroblast proliferation and accumulation of vast numbers of necrotic heterophils in caseous exudate. Bacterial colonies are often present in caseous exudate and contain numerous organisms. *E. coli* colonies have a typical appearance in histologic sections. They are usually circular with concentrated bacilli forming a distinct smooth perimeter with fewer bacilli and spaces centrally. They stain negative with tissue Gram stain. Lesions of predisposing respiratory disease are usually present in the trachea and lungs and consist of lymphoid follicles, epithelial hyperplasia, and epithelium-lined air passages that may contain heterophils. Pneumonia and pleuropneumonia are more common in turkeys while chickens usually have pleuritis or pleuropneumonia with less lung involvement. Extension of the disease process into the oviduct from the left abdominal air sac may occur and cause salpingitis in juvenile birds (Figure 18.1H).

Inoculating pathogenic *E. coli* or bacteria-free culture filtrates into the air sac readily reproduces lesions of uncomplicated coliform infection (22, 105). Airsacculitis occurs within 1.5 hours. Bacteremia and pericarditis develop within 6 hours. In birds that survive, lesions are well-developed by 48 hours postinoculation. Most mortality occurs during the first 5 days. Recovery is usually rapid if birds survive the initial infection, although a few with persistent anorexia become emaciated and die. Ask *et al*. (22) have developed a defined method for determining susceptibility to colibacillosis that is reproducible and has been used to determine the relative innate (genetic) susceptibility of various broiler chicken genotypes to the disease (21).

Enteric-Origin Colisepticemia. Enteric-origin colisepticemia is most common in turkeys. *E. coli* gain access to the circulation and tissues following damage to the intestinal mucosa by infectious agents. The most common predisposing agent is hemorrhagic enteritis virus (387, 416, 518). Usually only one or two types of *E. coli* are involved in the disease outbreak and those in the tissues and intestinal tract are the same.

Lesions are typical of the acute septicemic stage of colibacillosis (75). Affected birds are in good physical condition and often have full crops containing feed and water. The most characteristic lesions are congestion or green discoloration of the liver, marked enlargement and congestion of the spleen, and congested muscles (Fig. 18.2C). Microscopically the spleen is congested with proteinaceous fluid in sinuses and has multifocal necrosis, often containing intralesional bacteria. Fibrin thrombi are present in liver sinusoids and occasionally renal glomeruli. In some cases, multiple, pale foci in the liver are seen. Microscopically these are areas of acute necrosis initially, but with time they evolve into granulomatous hepatitis in survivors (Fig. 18.2D). After a few days birds eventually develop lesions similar to those of respiratory-origin colisepticemia.

Neonatal Colisepticemia. Chicks are affected within the first 24–48 hours after hatching. Mortality remains elevated for 2–3 weeks and usually totals 10–20%. Up to 5% of the flock may be stunted and require culling. Unaffected birds grow normally and the disease does not appear to spread. Initial lesions consist of congested lungs, edematous serous membranes, and splenomegaly. The proventriculus and lungs develop a dark color that can approach black as the interval between death and necropsy increases. Microscopically bacteria are numerous in affected tissues and easily identified. After a few days the typical pattern of acute, fibrinoheterophilic polyserositis involving the pericardial sac, pleura, air sacs, and peritoneum becomes evident. Lesions are often extensive and severe in birds that survive into the second week. Occasionally birds with arthritis or osteomyelitis may be found late in the disease. Most affected birds have yolk sac abscesses suggesting the navel is the portal of entry. Alternatively, *in ovo* infection may be responsible (348).

Layer Colisepticemia. Colisepticemia is usually a disease of young birds, but occasional outbreaks of acute *E. coli* infection resembling fowl typhoid or fowl cholera occur in mature chickens and turkeys (28, 108, 520, 560). Acute colibacillosis in layers is being seen with increasing frequency (520). The majority of outbreaks are associated with onset of egg production, but less frequently they occur at an older age, or may continue as the flock ages and potentially spread to older flocks on the same farm. The disease may reoccur in the same flock or subsequent flocks placed on farms or in houses where affected flocks had been previously (520). Death usually occurred suddenly without premonitory signs although depression and/or dirty vents were observed in some affected hens in approximately half of the flocks. Weekly mortality was significantly higher in affected flocks than age-matched control flocks (0.26–1.71% vs. 0.07–0.30%). Cumulative mortality ranged up to 10% and mortality remained elevated for 3–10 weeks. Polyserositis (perihepatitis, pericarditis) and peritonitis associated with free yolk in the peritoneal cavity were present in most birds at necropsy. Oophoritis and salpingitis occurred less frequently.

Isolates of *E. coli* from outbreaks in Italy were lactose-negative, nonmotile, and belonged to serogroup O111. Intramuscular inoculation of the O111 APEC reproduced the disease whereas it developed in only a few birds following oro-nasal administration (560). In contrast, the majority of isolates from outbreaks in Belgium were serogroup O78. Outbreak isolates were more likely to have P fimbriae (F11), especially if they were serogroup O78 and recovered from the heart. Serogroup O78 isolates also had the lowest percentage of motile strains (520). A number of virulence factors were significantly more frequent in outbreak isolates compared to control isolates. However, when cecal isolates and extra-intestinal isolates within either the outbreak or control groups were compared, they were not significantly different. Collectively no virulence factors or combination of factors were found only in outbreak isolates (523).

The pathogenesis of the disease is unknown, but stress associated with onset of egg production is believed to be an important contributing factor (560). Ascending infections via the oviduct have been suggested as a means by which *E. coli* gain access to systemic tissues, but in a recent study, higher colonization rates

of the trachea, but not the oviduct, in affected flocks suggests layer colisepticemia may be aerogenous (520). Lack of recognized stressors or indications of diseases known to predispose chickens to colisepticemia suggest layer colisepticemia results from a primary *E. coli* infection (520, 522).

Risk factors for developing layer colisepticemia include close proximity to other poultry farms and higher stocking density (521). Control has been through chlorination of water or treatment of the flock with appropriate antibiotics.

Coliform Septicemia of Ducks. Coliform septicemia of ducks is characterized by moist, granular to curd-like exudate of variable thickness causing pericarditis, perihepatitis, and airsacculitis. A characteristic odor is often noted at necropsy. Liver is frequently swollen, dark, and bile stained, and spleen is swollen and dark. *E. coli* (usually O78) can be recovered from any internal organ (302, 340). *Riemerella anatipestifer* causes similar lesions, but it can be identified by appropriate cultural procedures.

Coliform septicemia occurs throughout the growing season but is most frequent in late fall and winter. All ages of ducklings are susceptible. Distribution of losses suggests individual farms, rather than hatcheries, are the source of infection (302). Poor husbandry and marked contamination of pond water used by ducklings were contributing factors to an outbreak of colisepticemia in captive mallards (340).

Colisepticemia Sequelae. Death is the usual outcome of colisepticemia, but some birds may completely recover or recover with residual sequelae. If the bird does not control *E. coli*, it can localize in poorly protected ("immunologically privileged") sites including the brain, eyes, synovial tissues (joints, tendon sheaths, sternal bursa), and bones. In immature females, salpingitis can occur when there is involvement of nearby air sacs. IBV infection of the oviduct may also be an important predisposing factor in juvenile salpingitis. After *E. coli* is no longer present, constrictive pericarditis develops as the exudate in the pericardial sac undergoes organization, and liver fibrosis may result (Figure 18.1G). Ascites may develop because of residual pulmonary damage from combined *E. coli*–IBV infection (512, 552). It also is possible for ascites to develop from the direct action of endotoxin on the pulmonary vascular system. Endotoxin causes vasoconstriction leading to pulmonary hypertension and the potential to develop ascites (pulmonary hypertension syndrome) (72).

Meningitis. Escherichia coli localization in the brain is uncommon. Meninges are affected (meningitis) but in some birds there also is involvement of the brain (encephalitis) and ventricles (ventriculitis). Meningeal lesions are evident at necropsy as zones of discoloration adjacent to major blood vessels. Fibrinoheterophilic to heterophilic exudate is seen microscopically early in the infection; the lesion becomes more granulomatous with time. Bacteria are usually numerous within lesions but may not form distinct colonies.

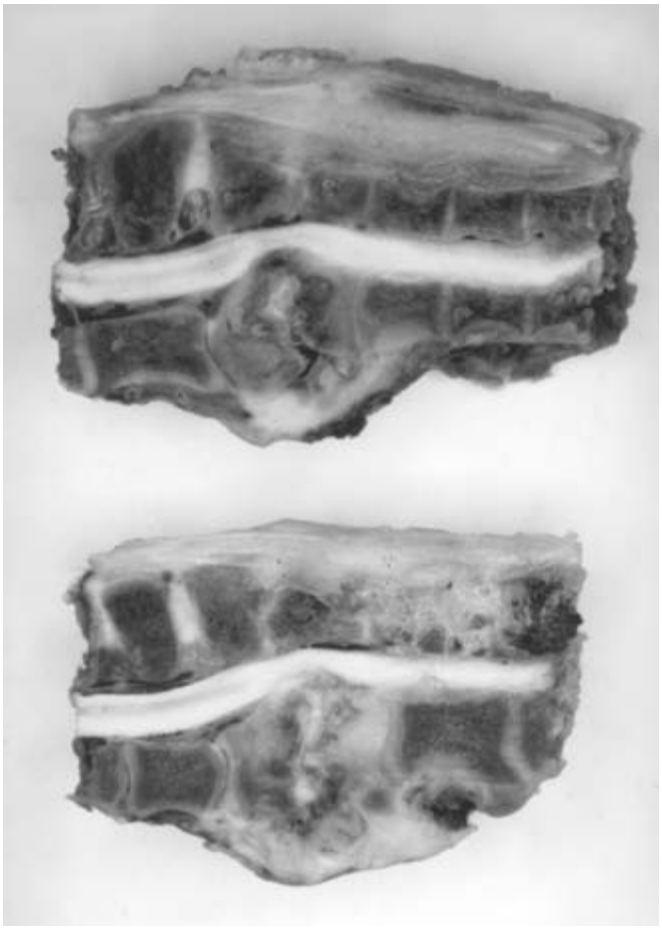
Panophthalmitis. As with the brain, involvement of the eye is uncommon. However, if it is infected the resulting panophthalmitis is severe (179, 372). Typically there is hypopyon and hyphema, and infection is unilateral (Fig. 18.2F). The eye is swollen, cloudy to opaque, and may be hyperemic initially. Later the eye shrinks as it undergoes atrophy. Fibrinoheterophilic exudate and numerous bacterial colonies are present throughout the eye. Inflammation, especially adjacent to necrotic tissue, becomes granulomatous with time. Varying degrees of retinal detachment, retinal atrophy, and lysis of the lens also may be seen. The organism persists in the diseased eye for long periods of time.

Osteoarthritis and Synovitis. Localization of *E. coli* in bones and synovial tissues is a common sequel to colisepticemia (Fig. 18.2E). The term osteoarthritis is used when a joint is inflamed and one or more bones making up that joint have osteomyelitis. Polyarthritis refers to involvement of more than one joint. Bacterial chondronecrosis with osteomyelitis (BCO) is another name that has been used (334). For a recent review see (334).

Affected birds likely have insufficient resistance to completely clear the bacteria. Hematogenous spread of *E. coli* following hemorrhagic enteritis virus infection of turkeys resulted in synovitis, osteomyelitis, and green liver discoloration (119). Intravenous inoculation to simulate hematogenous spread to bones and joints has been used to reproduce the lesions, but mortality from the initial septicemia is often high (37). A preferable method is to inoculate the air sac of birds with low numbers of *E. coli* after a pre-treatment with dexamethasone (222).

Mild to severe lameness and poor growth are seen clinically and affected birds are more likely to be victims of persecution ("cannibalism"). Often multiple sites are involved. Bacteria colonize the vascular sprouts that invade the physis of a growing bone provoking an inflammatory response that results in osteomyelitis. Transphyseal blood vessels in birds serve as conduits for the process to spread into the joint and surrounding soft tissues. Compared to clinically normal turkeys, lame turkeys had the following: higher splenic and liver weights, lower body and bursal weights, decreased cellular immunity, normal to increased humoral immunity, decreased circulating lymphocytes, increased circulating total leukocytes, monocytes, and heterophils, normal to marginally depressed phagocyte function, increased serum protein, uric acid, and blood urea nitrogen (BUN), and decreased hemoglobin, iron, alkaline phosphatase, and gamma-glutamyltransferase (38). Bones most often affected are the tibiotarsus, femur, thoracolumbar vertebra, and humerus (362). Proximal physes of long bones are more frequently affected than distal physes. Lesions typically form where endochondral ossification is occurring and extend proximally to involve the adjacent physeal cartilage. It is common to find both osteomyelitis and tibial dyschondroplasia together, but this is most likely due to their occurrence at the same location rather than a cause and effect relationship. Osteomyelitis is easily recognized on gross examination of bones opened to expose the physes, but small lesions that can only be seen microscopically also occur (334).

Hock, stifle, hip, and wing joints and articulations of the free thoracic vertebra are sites where arthritis is most likely to occur (362). Lesions in other joints are less common. Trauma to joints



18.4. Spondylitis involving articulating free thoracic vertebrae of two lame turkeys (fixed tissues). Pressure from the lesion on the spinal cord has caused demyelination of the ventral tracts. *Escherichia coli* is a common cause of this lesion, but other bacteria that can localize in bones and synovial tissues may also be a cause.

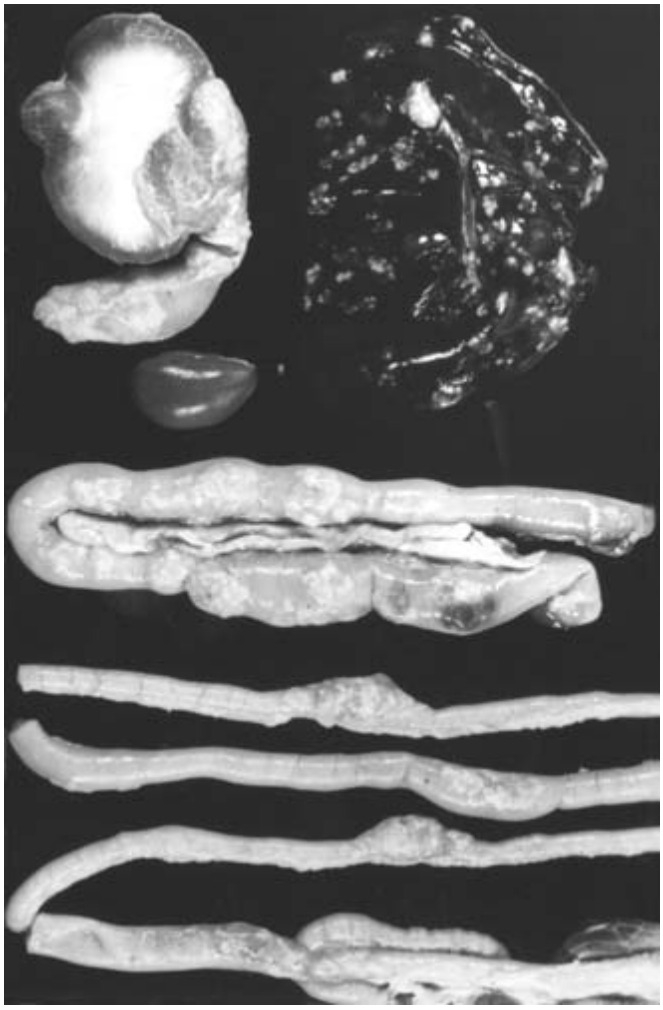
and growing bones may predispose to the development of lesions. Tenosynovitis frequently accompanies arthritis. Less commonly, spread of the inflammatory process from a joint into the periarticular tissues occurs. An infectious sternal bursitis is also common but must be distinguished from traumatic sternal bursitis in which fluid is seen but not exudate. When inflammatory lesions involve the shoulder joint or proximal humerus, extensive exudate can accumulate between the superficial and deep pectoral muscles (Figure 18.1E). Lesions that develop in joint spaces of the articulating free thoracic vertebra are characterized by spondylitis (spondylosis), which results in progressive paresis and paralysis (146)(Figure 18.4). Lesions in the distal articulation of the free thoracic vertebra occur more commonly than lesions in its proximal articulation or both articulations.

Turkey Osteomyelitis Complex. Turkeys with turkey osteomyelitis complex (TOC) have infectious, inflammatory lesions in bones, joints, or periarticular soft tissues and enlarged, green discolored livers that are used at processing to indicate the possible presence of intraosseous lesions (80, 119, 221, 222). Green liver discoloration is rarely identified in the field even in lame turkeys. Most often green discolored livers are detected at processing and result in downgrading and, depending on severity, partial or whole bird condemnation. It is likely that green discoloration of the liver is more evident in processed birds because they have bled out in contrast to birds in flocks that either die or are euthanized.

In TOC, osteomyelitis and arthritis occur as described above. Spondylosis of the articulating free thoracic vertebra can be associated with green liver discoloration (30). Not all birds with green discolored livers have TOC, but generally birds with TOC have discolored livers (80). Feed and water withdrawal do not cause green liver discoloration (229), but infection with *Mycoplasma synoviae* was associated with a high percentage of carcasses with green discolored livers in the absence of TOC (30). The Food Safety Inspection Service in the United States uses green discoloration of the liver at processing to identify carcasses that may have TOC so that any abnormal tissue can be removed from the food chain. Osteomyelitis lesions can also be detected by ultrasonography (361). Tom turkeys are more affected than hens and affected birds have decreased cell-mediated immunity (38, 222).

Escherichia coli are frequently isolated from the lesions (80, 119), but other bacteria may also cause TOC lesions, especially *Staphylococcus aureus*, or *S. hyicus* (503)(see Staphylococcosis). Culturing bones and livers from affected and unaffected birds in two turkey flocks resulted in recovery of pleomorphic, Gram-variable bacteria consistent with L-forms (cell-wall-deficient forms). Positive cultures were obtained more frequently from affected birds and bones than from unaffected birds or livers. The significance of these organisms in the disease is unknown, but the high number of isolates suggests these bacterial forms may be more common in turkeys than has been generally realized (36).

A laboratory model of TOC based on injecting turkeys with dexamethasone followed by inoculation of low numbers of *E. coli* into an air sac has been developed and used to study the pathogenesis of the disease (219, 220, 222, 223, 224). Dexamethasone injections alone will reproduce the lesions of TOC by increasing susceptibility of the birds to opportunistic bacteria, particularly *S. aureus.* Exposure of turkeys to low levels of *E. coli* via air sac inoculation increases the occurrence of TOC (222). The current hypothesis is that TOC is related less to the virulence of the infecting bacteria than it is to an inappropriate response of a subpopulation of male turkeys to stress, which increases their susceptibility to opportunistic bacterial infections. The greater susceptibility of turkeys selected for rapid growth and higher body weights to experimental TOC further supports the concept of genetic susceptibility that is most likely mediated by how the birds respond to stress (218). The protective effect of vitamin D_3 (221) also suggests a possible genetic basis for TOC susceptibility related to vitamin D receptors and their function (222). However, when vitamin D metabolites were administered, TOC was reduced as with vitamin D treatment, but there were toxic effects in dexamethasone-treated turkeys challenged with *E. coli* (225).



18.5. Coligranuloma in a market-age turkey. Numerous, nodular lesions are located in gastrointestinal tissues including liver, but they do not involve the spleen. A mucoid *Escherichia coli* was isolated.

Coligranuloma (Hjarre's Disease). Coligranuloma of chickens and turkeys is characterized by multiple granulomas in liver, ceca, duodenum, and mesentery, but not spleen (Fig. 18.5). The disease also has been described in quail (91). Coligranuloma is an uncommon form of systemic colibacillosis that usually occurs sporadically in individual birds, but can cause mortality as high as 75% when a flock is affected. Serosal lesions resemble leukosis tumors. Early in the disease there is confluent coagulation necrosis involving as much as half the liver. Only scattered heterophils are seen, and at the edge of the necrotic areas there are a few giant cells. Subsequently typical heterophilic granulomas are present in the affected tissues. Pyogranulomatous typhlitis and hepatitis, which may be related to coligranuloma, have been described in turkeys with cecal cores and ruptured ceca (354).

Pathogenesis of the Infectious Process

Escherichia coli enter host tissues following mucosal colonization or directly through breaks or openings in the skin. Mucosal colonization is dependent on adhesin factors that permit the bacterium to attach to receptors and subsequently reproduce. A variety of fimbrial and non-fimbrial adhesins are produced by *E. coli,* which facilitates their attachment to host cells (see Virulence Factors). Good evidence exists that two fimbriae—Type 1 (F) and P fimbriae—are important in the initial stages of infection. Type 1 fimbriae are expressed by *E. coli* that attach to upper tracheal epithelium (422), oviductal epithelium (346), and digestive tract mucosae (121). P fimbriae are expressed in deeper tissues (422). Type 1 fimbriae bind to mucus in the digestive tract but not to goblet cells producing the mucus. In contrast, AC/I fimbriae bind poorly to mucus but attach to goblet cells (121). Flagella aid in penetrating the mucous layer in order to reach the cell surface, and curli, another adhesin factor, aids in attachment to the cell surface (288, 289).

Virulent strains are capable of traversing the mucosa, especially if an injurious agent has compromised it, and surviving within the internal milieu of the body. Air sac epithelial cells round up following inoculation with virulent strains, which causes them to separate from each other providing bacteria access to systemic tissues (105). Toxins in cell-free culture filtrates, most likely endotoxin, produce the same acute inflammatory response as the living organism (105). Other toxins that could aid or provide the same capability have been demonstrated in APEC (see Virulence Factors), but their role in disease has yet to be determined.

Once *E. coli* becomes extramucosal, the environment it has entered is extremely hostile. Unless the organism is equipped with survival capabilities (*e.g.*, "virulence" factors), it is rapidly destroyed by phagocytic cells such as heterophils, thrombocytes, and macrophages (197, 198, 542). Macrophages located primarily in the spleen and liver phagocytize bacteria that gain access to the circulation (18). Complement and antibodies to O antigens (endotoxin), outer-membrane proteins (siderophores), and fimbriae serve as opsonins to promote phagocytosis and destruction of the organism (19, 20). Endotoxin also decreases the bacteriocidal ability of pulmonary macrophages (129), which may aid in survival and dissemination of *E. coli.*

Immediately after *E. coli* contacts host tissues, there is an acute inflammatory response. Acute phase proteins produced in the liver and cytokines IL-1, IL-6, and tumor necrosis factor increase rapidly following exposure to endotoxin or *E. coli*, which can serve as nonspecific indicators of early disease (70, 381, 550). Acute phase effects of endotoxemia include decreased feed consumption and efficiency, body and breast weight, and tibial bone size, weight, calcium content, and breaking strength, and increased mortality, liver weight, plasma ionized calcium, and antibody responses. Increasing amounts of endotoxin in the circulation decreases body weight and bone breaking strength (341). Vascular permeability increases and fluid and protein accumulate in the tissue. Serous membranes become wet and edematous and liquid begins to accumulate in body cavities. Chemotactic factors attract heterophils, which marginate in postcapillary venules and emigrate into surrounding tissues (321). Between 6 and 12 hours soft, gelatinous exudate becomes grossly visible. Heterophils can kill *E. coli* extracellularly by substances

such as ß-defensins released as they degranulate and die (198, 496, 497). After 12 hours there is a progressive shift in inflammatory cells from heterophils to macrophages and lymphocytes.

Exudate continues to accumulate and eventually undergoes caseation to form a firm, dry, yellow, irregular, cheese-like mass. Microscopically caseous exudate consists of heterophilic granulomatous exudate containing variable numbers of embedded bacterial colonies. A palisade of multinucleated giant cells and macrophages surrounds the exudate (75). Depending on the size of the mass of exudate, an extended period of time will be required for the exudate to be slowly eroded away by the action of the surrounding phagocytic cells. Viable bacteria persist as microcolonies within the exudate. Epithelial tissue may be restored if damage has not been too severe, but usually there is some degree of fibrosis, which may be complete (scarring) if tissue destruction has been extensive. Exudate containing fibrin may undergo organization eventually being converted to scar tissue. Gross lesions are inversely related to virulence. Highly virulent strains cause mortality so quickly that gross lesions have little time to develop, whereas birds infected with less virulent strains survive longer and develop more extensive lesions.

Diagnosis

Isolation and Identification of Causative Agent

Diagnosis is based on isolation and identification of *E. coli* from lesions typical of colibacillosis. Care must be taken to avoid fecal contamination of samples. Isolation of the organism from visceral organs of birds undergoing decomposition must be interpreted cautiously as *E. coli* rapidly spreads from the intestinal tract of dead birds. Bone marrow cultures are easy to obtain and are generally free of contaminating bacteria. Material should be streaked on eosin-methylene blue (EMB), MacConkey, or tergitol-7 agar, as well as non-inhibitory media. A presumptive diagnosis of *E. coli* infection can be made if most of the colonies are characteristically dark with a metallic sheen on EMB agar, bright pink with a precipitate surrounding colonies on MacConkey agar, or yellow on tergitol-7 agar. Strains of *E. coli* can be slow or non-lactose fermenters and appear as non-lactose-fermenting colonies. Definitive identification of *E. coli* is based on the organism's characteristics (see Etiology). A flow chart for the isolation and identification of *E. coli* has been published (301). A number of manual and automated systems are available for identification of bacteria, including *E. coli.*

Antigenic identification, determination of virulence factors, or fingerprinting of the isolate might be helpful, particularly when done as part of an epidemiologic investigation. The correlation between virulence and complement resistance suggests this may be a good method for screening isolates for possible disease association. A relatively simple rapid turbidimetric assay has been described (408). A constellation of 6 virulence genes (*sitA, iroN, hlyF, iss, iutA*, and *etsA*; see Table 18.3) that appear to characterize virulent strains have been identified in >74% of APEC isolates, but only occur infrequently in commensal strains. A multiplex PCR to detect them may provide a means to distinguish between commensal and pathogenic isolates by laboratory methods without having to resort to either embryo or chick lethality tests in the future (L. Nolan, unpublished data).

Serology has not been used as a diagnostic method. However survival after challenge correlated better with antibody titers detected by an enzyme-linked immunosorbent assay (ELISA) than by the standard indirect hemagglutination procedure (303).

Procedures to detect acute phase proteins (70, 341, 381, 550) or shifts in the heterophil/lymphocyte ratio (183) can serve as nonspecific markers of the inflammation and stress that accompany colibacillosis.

Differential Diagnosis

Acute septicemic diseases may result from pasteurellae, salmonellae, streptococci, and other organisms. *Chlamydiophila*, pasteurellae (*Pasteurella*, *Ornithobacterium*, *Riemerella*), or streptococci *(Streptococcus*, *Enterococcus*) sometimes cause pericarditis or peritonitis and other bacteria, mycoplasmas, and *Chlamydiophila* can cause airsacculitis. Many organisms including viruses, mycoplasmas, and other bacteria can cause synovial lesions similar to those resulting from *E. coli* infection. A variety of organisms including *Aerobacter* spp., *Klebsiella* spp., *Proteus* spp., salmonellae, *Bacillus* spp., staphylococci, enterococci, or clostridia are frequently isolated (often as mixed cultures) from yolk sacs of embryos and chicks (200). Liver granulomas have many causes, including anaerobic bacteria belonging to the genera *Eubacterium* and *Bacteroides.*

Intervention Strategies

Management Procedures

Fecal contamination of hatching eggs is the most important way that *E. coli* are transmitted between flocks. Collecting eggs frequently, keeping nest material clean, not using floor eggs, discarding cracked eggs or those with obvious fecal contamination, and fumigating or disinfecting eggs within 2 hours after they are laid can reduce transmission. *E. coli* on the shell surface can be reduced or eliminated with sanitizers (465). Application of sanitizers by electrostatic spraying improves efficacy (446). Ultraviolet irradiation can reduce or eliminate *E. coli* and other bacteria on the surface of hatching eggs without altering conductance or hatchability (87). If infected eggs are broken during incubation or hatching, the contents are a serious source of infection to other chicks, especially when personnel and egg-handling equipment are contaminated. Eggs are particularly susceptible just before hatching. Methods for preventing incubator and hatcher dissemination are unknown. However, venting incubators and hatchers to the outside and having as few breeder flocks as possible represented in each unit will help reduce cross-contamination and losses. Chicks are colonized by strains of *E. coli* that they are exposed to in the hatchery from people or the environment and take the organisms with them when they are placed on the farm. Contaminated chicks survive better if kept warm and they are not deprived of food for an extended period of time.

Survival tends to be better in birds fed high protein diets, increased selenium (300), and increased vitamins A and E (226, 390, 504, 505). However, high levels of vitamin E can be detri-

mental to resistance to coliform cellulitis, colibacillosis, and antibody production (145, 307, 319, 464). Response to vitamin E is likely interrelated with the genotype of the bird (470, 554). Feeding can have an impact on severity of colibacillosis. Chickens fed on alternate days were more resistant to *E. coli* challenge than full-fed chickens (54, 423).

There are no known methods for reducing the level of pathogenic *E. coli* in the intestinal tract and feces, although consideration that 1) pelleted feed has fewer *E. coli* than mash, 2) rodent droppings are a source of pathogenic *E. coli,* and 3) contaminated water can contain high numbers of the organism should not be overlooked. Hot pelleting processes destroy *E. coli* (125), but care must be taken not to recontaminate finished feed. Adding 5–10% egg yolk powder to feed effectively reduced or eliminated *E. coli*, and other bacterial foodborne-illness pathogens in layers (270). Commercial broiler chickens and breeders were less productive when their water contained *E. coli* and nitrates (177, 178). Chlorination of drinking water and use of closed (nipple) watering systems have decreased the occurrence of colibacillosis and condemnations for airsacculitis (55, 108, 405). Pathogenic strains of *E. coli* can be competitively excluded from intestines of chicks by seeding them with native microflora from resistant chickens (535), commercial competitive exclusion products (194, 216), or *Bacillus subtilis* spores (290, 506). A similar effect was achieved following *in ovo* inoculation of *Lactobacillus reuteri* (122). Food and water deprivation increased the occurrence of spontaneous bacteremia (306).

Infection with *M. gallisepticum* and/or IBV overcomes the protective effect of native flora colonization (536). Raising mycoplasma-free birds and reducing exposure of birds to viruses causing respiratory diseases can reduce *E. coli* infection of the respiratory tract of birds. Effective vaccination to protect against respiratory tract pathogens and immunosuppressive agents that predispose to colibacillosis reduces occurrence of the disease (328).

Birds acquire nonspecific resistance to colibacillosis following moderate stress and socialization with people. Nonspecific resistance is short-lived and can be minimized by cold stress or corticosterone (326).

Maintaining good air and litter quality is fundamental to reducing risk of a flock developing colibacillosis (96). Fluctuating between cool and warm temperatures and modulating airflow have been used experimentally to produce colibacillosis in commercial chickens (245). Proper ventilation minimizes respiratory tract damage from ammonia and reduces the levels of bacterial and aerial endotoxin exposure. Ammonia, even at levels below those that can be detected by human smell, impairs mucociliary clearance of inhaled particulates (367, 397). Degree of damage to the respiratory mucosa correlates with ammonia exposure (366). Dust also increases the risk of colibacillosis (202). Bacteria preferentially adhere to dust particles because of electrostatic charges. The combination of dust and ammonia results in birds inhaling high numbers of bacteria and being unable to clear them from their respiratory tract.

Wet litter provides an excellent environment for *E. coli* to persist and reach high numbers. Higher numbers of *E. coli* and *Salmonella* were found in litter that had water activity >0.9% and moisture content >35%. Air velocities across the surface of the litter of at least 100 ft/min produce drier litter and decrease the number of *E. coli* (431). Airflow across litter may not be uniform throughout a commercial broiler house. Low airflow correlated with high coliform counts in litter and litter with high numbers of coliforms was 16 times more likely to be in a low airflow area of the house (364). The incidence and severity of footpad dermatitis at processing can be used as an indicator of litter condition and air quality that were present during the production cycle (206). Maintenance of waterers is essential in eliminating wet spots in the house. Daily raking of soiled litter that builds up around feeders and waterers, removing heavily soiled litter, tilling, and replacing or covering wet litter with fresh dry litter are useful procedures for maintaining good litter quality.

Vaccination

Types of Vaccines

A variety of vaccines and vaccination methods have been developed including passive and active immunization, use of inactivated and live products, recombinant and subunit vaccines, and immunization against specific virulence factors. Most have not gone beyond the experimental investigation phase. No vaccination procedure has proved to be highly efficacious in commercial production and no product is used widely in the industry at the present time. A commercial vaccine containing F11 (PapA) fimbrial antigen and flagellar antigen (FT) is licensed in Europe for use in broiler breeders to provide natural passive immunity to progeny of vaccinated hens (Noblis E. coli Inac, Intervet).

Inactivated Vaccines. Effective inactivated vaccines against various serotypes including O2:K1 and O78:K80 have been produced (19, 68, 100, 101, 513). They provide protection against the homologous serogroups, but no significant cross-protection against heterologous serogroups. An inactivated O78 vaccine protected ducks (459). Both homologous and heterologous protection was provided by a vaccine prepared by ultrasonic inactivation of the organism followed by irradiation (336). A vaccine containing bacterial membrane vesicles was effective in protecting turkey poults against challenge with pathogenic *E. coli* by stimulating antibody production, bacterial-lysis activity of complement, T cell proliferation, and cytotoxic T cell activity (69). Multivalent vaccines made from pili containing low levels (180 μg) of protein per dose reduced the severity of challenge infection (193). Absorbed sera indicate pili of serotypes O1, O2, and O78 are antigenically different (499).

Live Vaccines. A live vaccine prepared from a naturally occurring, nonpathogenic, piliated *E. coli* strain (BT-7) was efficacious when used in chickens older than 14 days of age. Protection against both homologous and heterologous strains was demonstrated (147). *E. coli* J5, a mutant strain that has incomplete endotoxin in the cell wall exposing Gram-negative core antigen, was both safe and effective for protecting chicks (3, 4). Antibody titers to Gram-negative core antigens that develop in commercial chickens peak during the pullet period (445).

Recombinant and Mutant Vaccines. A *carAB* mutation of a virulent O2 serotype caused defective utilization of arginine and pyrimidines, increasing the requirements by the mutant. As low levels of these substances are generally available *in vivo*, the organism was unable to sustain itself, which resulted in a self-limiting infection. The mutant strain was found to be stable, immunogenic, and attenuated. Turkeys orally vaccinated with the mutant were protected against colibacillosis in a hemorrhagic enteritis virus-parent wild-type strain challenge model (286). Mutant O2 and O78 APEC with deletions of the genes *cya* or *crp*, which are involved in energy production, were used as a spray vaccine to immunize broiler chickens. The mutants were safe and immunogenic but provided only limited protection against airsacculitis following challenge (407). Similarly, strain O78 mutants with deletions of *galE*, *purA*, and *aroA* genes were found to be safe and immunogenic, but provided only moderate protection against homologous challenge with no protection against heterologous challenge (266).

In contrast to high mortality caused by the parent and wild-type strains, attenuated streptomycin-dependent (str-dependent) mutants derived from a virulent APEC did not cause mortality in challenged birds. No protection against cellulitis or systemic lesions resulted when birds were vaccinated with high numbers of the mutant strains by aerosol and oral routes. However, systemic lesions were significantly reduced when birds were given three vaccinations on days 1 (aerosol), 14 (oral), and 28 (oral) (13).

A recombinant vaccine using *Salmonella typhimurium* was constructed to produce homologous group B determinants and *E. coli* O78 antigen. Vaccinated birds seroconverted and were protected against subsequent challenge with a pathogenic *E. coli* O78 strain (437). A similar vaccine constructed to express *E. coli* type 1 fimbrial antigen in addition to O78 LPS provided protection against homologous challenge. The O78 LPS was responsible for most of the efficacy of the vaccine although presence of the fimbrial antigen did decrease the severity of air sac lesions. The fimbrial antigen did not provide cross-protection following challenge with O1 and O2 APEC serogroups (438).

A preliminary study on immunization of chickens with Iss, a surface protein common to APEC but not commensal *E. coli* that is important in complement resistance, suggests the potential to achieve cross-protection among different serotypes (317).

Fimbrial vaccines containing FimH, the adhesin of F1A (type 1) fimbriae, or PapGII, the highly conserved portion of P fimbrial adhesion, were immunogenic but did not provide protection against APEC challenge (526, 527). The results of PapGII immunization differ from the finding that passive immunization with PapG yolk-derived antibodies was protective (see Passive Immunization).

Passive Immunization. Passive immunization results in increased resistance to aerosol challenge and clearance of bacteria from blood (363). Use of inactivated vaccines in breeders provided passive protection against homologous challenge in progeny, which was complete for 2 weeks and partial for several additional weeks post-hatch (209, 442).

Antiserum prepared in rabbits against iron-regulated outer membrane proteins of *E. coli* protected turkeys against mortality following challenge. Frequency of bacteremia at 96 hours after challenge, recovery of *E. coli* from air sacs, and severity of gross lesions were significantly reduced in immunized birds compared with control birds given normal rabbit serum or saline solution (58).

Antibodies extracted from the yolk of eggs laid by immunized hens provided homologous protection against an O78 APEC. Partial protection against heterologous challenge with O1 and O2 serotypes was provided by immunizing hens with P pilus adhesin (PapG) or the aerobactin outer membrane receptor IutA. Immunizing with PapG provided the best overall protection. Breeder hens immunized by this method may provide immunity to their progeny (265).

Immunopotentiation. A problem with recombinant vaccines is low immunogenicity, which could potentially be solved by using effective immunopotentiators. Inoculation of chickens by intramuscular or subcutaneous routes and chicks by intramuscular or *in ovo* routes with cytosine-phosphodiester-guanine (CpG) oligodeoxynucleotides improved livability and reduced cellulitis lesion size following challenge with APEC (169, 170). CpG motifs are present in high numbers in bacterial DNA and enhance innate immune responses (24, 170). Modification of *E. coli* heat-labile enterotoxin (LT) resulted in a nontoxic protein (nLT) that stimulated antibody production in chickens following either oral or parenteral co-administration of an antigen to chickens (528).

Treatment

Antimicrobial Drugs

Antimicrobial drugs have been used extensively for reducing losses from colibacillosis since their first introduction for treatment of poultry in the mid-1950s. Occurring in parallel with use of an antimicrobial has been a progressive development of resistance, which was initially identified following introduction of tetracyclines (479). Antimicrobial resistance is determined genetically and is usually transferable within a species or between different types of bacteria via mobile genetic elements—plasmids, integrons, and transposons (34, 299, 553). The greatest reservoir for transferable antimicrobial resistance factors in the poultry flock environment is not *E. coli* or even Gram-negative bacteria, but gram-positive bacteria that comprise over 85% of the bacteria in poultry litter (382). The intestinal tract of the chicken is a suitable environment for transfer of genes from tetracycline-resistant to susceptible *E. coli* strains. Adding tetracycline to the chick's drinking water accelerates the process. Resistance to other antimicrobials is co-transferred along with tetracycline resistance (205).

Growing concern over antibiotic resistance, especially multidrug resistance, and the potential of bacterial strains affecting people acquiring transmissible resistance factors from bacteria in animals (see Public Health Significance), has led to changes in the way antimicrobials are used to treat colibacillosis in poultry (473). Additionally, new antimicrobials are not being developed for use in poultry and the ones that have been used previously have lost much of their efficacy due to acquired resistance.

Recently, fluoroquinolones became available in the United States and elsewhere for treatment of colibacillosis in poultry, which generally proved to be highly efficacious (165). However, societal concerns about resistance in some strains (39, 164, 252, 272, 540), development of cross-resistance among different quinolones, and importance of this class of antibiotics for treating people have led to their withdrawal for use in poultry in many countries including the United States. For a review of antimicrobial resistance of avian *E. coli* see (541).

When selecting an antimicrobial to use for treatment, it is important to determine the susceptibility of the isolate involved in the disease outbreak so that ineffective drugs can be avoided. APEC frequently are resistant to tetracyclines, sulfonamides, ampicillin, and streptomycin (339, 525, 541, 553). Multiresistance is common (525) and may occur in conjunction with virulence factors (255). Numerous recent studies on antimicrobial resistance of *E coli* isolates from chickens (12, 51, 157, 273, 299, 339, 349, 388, 517, 553, 561), turkeys (10, 86, 453, 517), ducks (344, 533), eggs (360), and poultry feed and ingredients (324) have been done in different geographic areas. All showed some level of resistance in APEC and commensal strains, but there were regional variations. Most *E. coli* isolates from shell eggs were susceptible to all antibiotics (360). Occasionally resistance is higher among commensal strains compared to APEC, *e.g.*, ampicillin resistance in turkeys (10) but, in general, resistance tends to be greatest in APEC strains. A high percentage of *E. coli* isolates from turkeys are resistant to gentamicin, which has been attributed to the widespread use of day-old gentamicin injection (10). Gentamicin resistance among chicken-origin *E. coli* was associated with significantly greater virulence in an embryo lethality assay (349).

Even a highly effective drug may not result in improvement of the flock if too little is used or it is incapable of reaching the site of infection. Therefore, underdosing may promote development of resistance. Chicks given feeds with increasingly lower concentrations of ampicillin (1.7 and 5 g/ton) developed resistance that was directly correlated to the amount of antibiotic in the feed (8). Paradoxically, certain antimicrobials and anticoccidials commonly used at subtherapeutic levels in poultry for growth promotion and coccidiosis control inhibited transfer of a plasmid that is responsible for multiresistance in *E. coli*. The basis for the inhibition was attributed to the ion-binding properties of the drugs and interference with DNA uptake channels in the organism (325). Selective pressure from exposure to an antibiotic is not always essential for resistance to develop (73). Although resistance generally occurs following prior contact with an antimicrobial, it can occur naturally in the absence of previous exposure. Resistance to florfenicol and chloramphenicol, which had never been used in poultry in the United States, was found in *E. coli* isolates from chickens in the southeastern United States (271).

Water administration of apramycin proved effective in reducing the numbers of organisms in the digestive tract and preventing bacteremia in chickens (306). Neomycin reduced mortality in turkey poults exposed naturally to litter from flocks with colibacillosis (323).

Anticoccidials also have antimicrobial activity that may be beneficial in the prevention and treatment of coliforms. Monensin reduced colonization of chickens with *E. coli* O157:H7 to undetectable levels 14 days post-exposure compared with nonmedicated controls and chickens receiving other coccidiostats (487). In a recent study TAMUS 2032, a cationic amphipathic peptide antibiotic produced by *Brevibacillus texasporus*, improved performance and reduced mortality when added to the feed of commercial broilers with or without monensin following natural environmental challenge. Improved livability and productivity also resulted from adding monensin with or without bacitracin. Bacitracin alone provided no protection against colibacillosis (245).

Other Treatments

The declining use of antibiotics for prevention and treatment of colibacillosis has stimulated interest in alternative methods including prebiotics, probiotics, enzymes, digestive acidifiers, vitamins, immune enhancers, anti-inflammatory drugs, and other antimicrobial products. Although prebiotics and probiotics are widely available for use in poultry, few studies on their effect on colibacillosis have been published. Colonization with *E. coli* begins immediately after hatching making early administration of probiotics essential (122). Administration of a bacteriocin-producing strain of *Lactobacillus plantarum* F1 or the purified bacteriocin provided chicks protection against challenge with an O2 APEC. Fermentation of coarsely ground wheat with a mixture of *L. plantarum* and *Pediococcus pentosaceus* completely eliminated *E. coli* when the pH of the mixture was <4.0 for at least 24 hours (353). *L. johnsonii* significantly reduced colonization of the small intestine with *E. coli*, but had no effect on colonization of the ceca or large intestine (294).

In addition to lactobacilli, other microbes can inhibit colonization with *E. coli.* Specific strains of *Bacillus* spp. inhibit *E. coli* colonization in the digestive tract and have potential use as probiotics (27, 290). Formation of highly resistant spores simplifies administration of these bacteria to commercial flocks through feed. Extracts prepared from *Bifidobacterium* enhanced resistance to colibacillosis following oral administration. Cell-mediated immunity was enhanced in the treated chickens (276). Inoculating young birds with nonspecific competitive exclusion products derived from healthy adults reduced intestinal colonization by APEC (216).

Essential oils often have substantial inhibitory effect on *E. coli in vitro* (196, 409) and in the lower intestinal tract of chickens (242). A commercial oregano oil product has been used in organic poultry production, but there are no studies on its effect on colibacillosis. Isopathic (immune enhancing) and homeopathic treatments with multiple products were unsuccessful in modifying the response of 8-day-old broilers challenged intratracheally with an O78 APEC strain (529).

Bacteriophage administration provides another possible alternative to antibiotic medication for controlling colibacillosis (32, 62). Two bacteriophages isolated from municipal wastewater that lysed an O2 APEC were effective in reducing mortality from experimental colibacillosis caused by the homologous APEC strain when high numbers of phage were mixed with the inoculum,

given as a spray up to 3 days prior to challenge, or inoculated intramuscularly up to 48 hours post-infection (230, 231, 232, 233, 235, 236). Combination of bacteriophage treatment with enrofloxacin had a synergistic beneficial effect (234). While bacteriophage treatment has been shown to be efficacious, several challenges remain before a commercial product might be realized (235).

Several studies have shown vitamin E supplementation to have both prophylactic and therapeutic benefits for *E. coli* infections (226), but not all studies support this conclusion (see Management Procedures). Differences in results likely are due to differences in experimental designs, especially severity of challenge and the timing and manner in which vitamin E is administered. Use of aspirin or sodium salicylate reduced the impact of experimental colibacillosis in turkeys (226) and chickens (311) respectively. However, if used in high levels or in combination with other products that impair the inflammatory response, a reverse response can occur (226). Feeding a beta-glucan product obtained from yeast cell walls improved the response of chickens to *E. coli* challenge, but also depressed growth of unchallenged controls (228).

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Coliform Cellulitis (Inflammatory Process)

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Introduction

Coliform cellulitis, also known as avian cellulitis, inflammatory process, infectious process, or IP, is caused by *Escherichia coli* and characterized by sheets of serosanguineous to caseated, fibrinoheterophilic exudate in subcutaneous tissues. Lesions, often referred to as plaques, are located in the skin over the abdomen or between the thigh and midline (see Figures 18.2H, 18.6, and 18.7). Other colibacillosis lesions or reduced productivity occasionally may accompany coliform cellulitis (11, 15, 38, 51), but usually lesions are discovered at processing when inspectors open the thickened yellow abdominal body wall of an otherwise normal carcass. Interactions among *E. coli*, the birds, and their environment contribute to the disease.

Coliform cellulitis has emerged as a significant disease since its description in 1984 (42) because of increased condemnations, downgrading at processing, and higher labor costs to process affected flocks. Between 1986 and 1996, condemnations for coliform cellulitis increased almost 12-fold in Canada. In 1996, 0.568% of all birds processed and approximately 30% of total condemnations were classified under cellulitis (24). In an Ontario study conducted in 2001, 1.11% of birds processed in federally inspected slaughter plants were condemned for cellulitis (49). Estimated annual losses to the U.S. broiler industry due to coliform cellulitis have increased from $20 million in 1991 to more than $80 million in 1998 (47).

Etiology

Escherichia coli is the most frequently isolated organism from cellulitis lesions and is considered the cause of coliform cellulitis in chickens. Other bacteria have been found (*Pseudomonas aeruginosa, Proteus vulgaris, Enterobacter agglomerans, Pasteurella multocida, Streptococcus dysgalactiae, Aeromonas, Staphylococcus aureus, Actinomyces pyogenes,* etc.) but are not believed to be significant (4, 12, 29, 31, 42, 48, 52). *E. coli* isolates from cellulitis lesions are of the same serogroups as those from birds with other forms of colibacillosis and usually produce colicin and aerobactin (40). Virulence properties and molecular characteristics are similar among isolates from cellulitis and coliseptic lesions and normal birds (13, 19, 30). However, iso-



18.6. Coliform cellulitis lesion at processing. Skin over the abdomen is thickened and has a yellow discoloration. Carcass is normal except for the skin lesion.



18.7. Coliform cellulitis lesion at processing. A caseous sheet of exudate, often referred to as a plaque, is located in the subcutaneous tissues beneath an area of thickened, yellow discolored skin.

lates from cellulitis lesions have a greater ability to produce cellulitis in experimentally exposed birds than *E. coli* isolates from airsacculitis lesions or feces of healthy chickens (5, 21, 41). Cellulitis-type and systemic-type strains of avian pathogenic *E. coli* can be selected that cause a predominance of either cellulitis or septicemic lesions, respectively (18). Leclerc *et al*. (2003) suggested that greater adhesive capacity of some *E. coli* strains to deep tissue layers may explain higher cellulitis prevalence in affected flocks.

A vacuolating cytotoxin produced by cellulitis *E. coli* isolates is also produced by isolates from chickens with colisepticemia and swollen head syndrome but not by isolates from healthy chickens. The cytotoxin is similar to one produced by *Helicobacter pylori*, except that *H. pylori* cytotoxin is specific for mammalian cells, and the avian *E. coli* cytotoxin is specific for avian cells (44).

Initially, isolates of *E. coli* from litter and lesions could not be differentiated based on biotyping, suggesting that the litter was the source of *E. coli* implicated in cellulitis lesions (10). But genotyping has shown that the prevalence of pathogenic *E. coli* in a broiler house is independent of the prevalence of other *E. coli* in the environment (20). DNA fingerprinting identified the presence of endemic populations of specific cellulitis-associated *E. coli* that exist in the broiler house environment. These organisms persist for at least 6 months irrespective of partial or complete cleaning and disinfection and cause coliform cellulitis in successive flocks (47, 48).

Epidemiology

Regional differences in the prevalence of coliform cellulitis emphasize the important roles of environmental and management factors in the occurrence of the disease. Increased condemnation rates due to coliform cellulitis during the past 15 years indicate that changes have occurred in either the incidence or characteristics of risk factors associated with this disease over the same period. The most notable change during this time has been in the genotype and phenotype of the bird being raised, so it is not surprising that bird-related factors also contribute significantly to the incidence of scratches and subsequent coliform cellulitis.

Risk Factors

Breed

Fast-growing, heavy broiler strains are more likely to have an increased prevalence and severity of skin scratches, which predispose to coliform cellulitis. Several reasons may explain this association. The strength of the skin in broilers is related to genetics. The lack of association between scratches and abdominal circumference suggests that strain of bird *per se* could be a better predictor than body characteristics (9). Aggressiveness or nervousness of chickens may also be strain dependent. Birds from a more nervous strain could be more active, increasing the chances of being injured or scratched. If aggressiveness is a problem, the source could be farm dependent (e.g., behavioral studies have demonstrated the importance of socialization of the flock by the grower on the birds' behavior) (6). Rapid growth by modern broiler breeds results in higher stocking density sooner in the life of the flock, at a time when feathering is not yet well developed. Poor feathering and crowded conditions could have a significant impact on the incidence of coliform cellulitis. Macklin *et al*. (2002) showed that the major histocompatibility complex affects the likelihood of an individual chicken developing cellulitis, although not the severity of the lesion. Breed differences in immune response, feathering, and body conformation are known to exist, but their possible contribution to the occurrence of coliform cellulitis requires further studies.

Feathering

Feather cover helps protect the skin from damage. A positive association exists between scratches and poor feathering (8). Birds

with poor feathering have more abdominal skin exposed for a longer period of time than birds with good feathering. Although little is known about the nutrition and environmental factors that affect feather growth and development, birds kept in warm temperatures tend to feather less rapidly than birds kept in lower temperatures.

Sex

Coliform cellulitis occurs more frequently in males than females (7, 51). The gene responsible for sexing regulates feather growth. Slower feathering males may be more vulnerable to skin injuries because of greater exposure of the skin to potential physical damage. Sex may also contribute to coliform cellulitis because of its association with weight, aggressiveness, or management practices. Also, production time is longer for roasters than for broilers.

Stocking Density

Stocking density plays a dual role as a risk factor. It leads to an increase in skin scratches (8) and stress, but it also contributes by increasing the level of contact between birds. Cellulitis lesions occurred more readily when birds were palpated daily to simulate close contact among birds (29).

Litter

Flocks grown on straw were 2.8 times more likely to experience coliform cellulitis than flocks grown on shavings (7). Physically, straw consists of sharp, pointed pieces that may inflict minor injuries to the skin and predispose birds to infection. Straw may also provide a good medium for growth and multiplication of *E. coli* because of its ability to hold more moisture than shavings. A positive association also exists between the number of flocks raised on the same litter and cellulitis (46). However, this association could not be explained by an increase in litter bacterial load. Furthermore, litter environmental variables (water activity, pH, moisture content, and ammonia levels), as measured in this study, were also not significant. Nevertheless, litter quality should be considered an important factor by those working on reducing this condition in the field.

Total Down Time

Total down time is negatively associated with coliform cellulitis (i.e., the longer the down time, the lower the incidence of the disease) (2, 46). This supports the hypothesis that the bacterial load of the environment is associated with disease prevalence.

Ambient Air Temperature

In a prospective study, Schrader *et al*. (2004) found a positive association between ambient temperature during early grow-out and cellulitis. Their predictive model, after controlling for other significant variables, indicated a 40 to 60 percent increase in cellulitis as temperatures increased over a range of approximately 60°F (15.5°C) from 29°F (–1.7°C) to 94°F (34.4°C). Hence, low cellulitis prevalence flocks would increase from 0.5 to 0.8 percent, while high prevalence flocks would go from 1.2 to 1.9 percent.

Relative Humidity

Similar to ambient temperature, increased relative humidity at mid–grow-out correlated with increased occurrence of cellulitis. An increase in relative humidity from 36 to 93 percent was predicted to increase cellulitis from 0.3 to 0.9 percent in low-prevalence flocks and from 1.0 to 1.9 percent in high-prevalence flocks (46).

Feed

A positive association was observed between coliform cellulitis and feed company in a prospective study (7). The effect of nutrition on the pathogenic process of the disease is not well known. Amino acid levels in the feed may be important. Feed deficient in cysteine and methionine can cause nervousness and affect feathering (39, 45). A relative deficiency occurs in feeds with high energy to total protein ratios. High-energy feeds may also contribute to coliform cellulitis by increasing fat deposition in the skin, which may result in the skin being more susceptible to scratches and injuries (45).

Occurrence of coliform cellulitis was higher in vegetarian broilers compared to broilers fed feeds containing animal products. Condemnation rates for birds fed a standard diet, which contained growth promotants, antibiotics, and anticoccidials, was substantially lower (0.26 percent) than for birds fed a vegetarian or organic feed without additives (1.18 percent) (17).

Providing vitamin E at 300 mg/kg or vitamin A at 60,000 IU/kg improves the resistance of 6-week-old broilers against *E. coli* infection (50). Supplementation with vitamin E had a variable impact on the development of coliform cellulitis. Intermediate levels were superior to both lower and higher levels of the vitamin (27). Birds fed both vitamin E at 48 IU/kg and a zinc-protein complex at 40 ppm of zinc decreased the occurrence of coliform cellulitis (36). Improved wound healing and immune system potentiation by the supplements were considered responsible for the beneficial effect.

Pathobiology

Natural and Experimental Hosts

Coliform cellulitis affects chickens. Older chickens are more likely to develop lesions of cellulitis following inoculation of scratches or subcutaneous injection than young chickens, which tend to develop systemic disease and experience high mortality (14, 21, 22, 26, 33, 35, 41). Recently, a case in quail was described (2). Cellulitis also has been used to describe a skin disease that has recently emerged in turkeys. In Canada, on average, 0.37 percent of turkeys were condemned for cellulitis in 2005. In February 2006, the condemnation rate rose to almost 0.60 percent in some regions (3). However, the validity of this assessment has not been done, and several cases could be more consistent with gangrenous dermatitis. In late 1999, Gomis *et al*. (2002) identified 0.14 percent of about 27,000 birds examined at a slaughter plant as having cellulitis in legs or caudal thoracic area. These lesions were further categorized as cellulitis with open skin and no open skin lesions. Bacteria could be isolated from only about half the lesions. When present, *E. coli* was only pres-

ent in low numbers, often in association with other bacteria, such as *Proteus mirabilis, Lactobacillus* spp., *Klebsiella* spp., and *Staphylococcus* spp.

Pathology

Cellulitis lesions are primarily unilateral and located on the abdomen or thigh. Skin color varies from normal to yellow or red-brown, and the skin may be swollen at the site of inflammation (Fig. 18.6). The size of the lesion normally varies between 1–10 cm. (11). Scratches and scabs on the skin overlying the lesions often can be identified. Beneath the skin, there is subcutaneous edema, exudate, and muscle hemorrhage. A fibrinous to caseous plaque between the muscle and the subcutis is the characteristic lesion (Figs. 18.2H, 18.7).

Lesions develop rapidly; exudate is visible as early as 6 hours postinfection, and the caseous plaque could be experimentally produced within 18–24 hours postinfection. Rapid lesion development suggests that events occurring late in the life of the flock could be important in the development of lesions found at processing (14, 33). When birds were inoculated experimentally with *E. coli* strains isolated from coliform cellulitis lesions, the highest percentage of birds developing typical lesions had been challenged only 3 days prior to processing (32). Lesions were still present 3 weeks postinoculation (33).

High coliform cellulitis condemnation rates are not of hatchery origin. Scratching the dorsal skin surface of older birds and inoculating them with *E. coli* produces lesions referred to as type I coliform cellulitis, which have been thought to originate from navel infections in the hatchery. Bacteria and inflammatory exudate gravitate from the area where it originates to around the navel, producing the so-called type I lesion (34). In Canada, only 1.7 percent of coliform cellulitis lesions were consistent with a primary navel infection (11).

Experimental exposure of young chickens to cellulitis isolates of *E. coli* results in septicemia, death, or marked stunting, indicating that most birds affected by *E. coli* in the hatchery would either die or be culled before reaching the processing plant (21, 22, 35). No association between cellulitis and the source of eggs, age of parent flocks, total bacterial count, and coliform count in the hatchers was found (10).

Pathogenesis

Skin trauma, especially scratches, provides the main portal of entry into the host for specific cellulitis-type *E. coli* present in the litter. Applying bacteria to feather follicles from which the feather had been pulled did not cause coliform cellulitis. Oral feeding or swabbing the navel of young chickens did not produce cellulitis but did result in mortality, depressed growth, and other types of colibacillosis, which was dose dependent (22). The disease is reproduced readily by swabbing damaged skin with broth cultures or subcutaneous inoculation (14, 21, 22, 26, 33, 35, 41).

A recent study by Olkowski *et al*. (2005) compared a strain of fast-growing broiler chickens to a strain of leghorns. They concluded that the broiler strain was more predisposed to cellulitis because of an inferior first line of defense of their skin. Indeed, compared to leghorns, the wound-healing process was slower; the lesions were more severe and covered a larger area; and the mobilization and functionality of phagocytic cells were inferior.

Usually, an affected bird has only skin lesions, but concurrent lesions of systemic colibacillosis occasionally can be found, suggesting that cellulitis may result from systemic spread or, conversely, that localized lesions in the skin can be a source for systemic disease. The latter is inversely correlated with age (i.e., the younger the bird, the more likely it is to develop systemic disease) (14, 21). Lesions have been correlated with other categories of condemnation in which *E. coli* would be expected to play a significant role (septicemia, airsacculitis, etc.) (10, 11, 13, 15, 51). Coliform cellulitis has been associated with previous outbreaks of colibacillosis in some flocks (40).

A positive association between cellulitis and ascites has been shown, but in one study, it could only account for 10 percent of coliform cellulitis cases (10, 51). Ascites is a common condition in broiler chickens characterized by an abnormally large abdomen. Because most cellulitis lesions are located in the abdominal area, it may be that ascites is a biological predisposing factor for cellulitis. Also, it is possible that both conditions may share common risk factors such as rapid growth.

Valgus-varus leg deformity, characterized by lateral or medial deviation of the distal tibiotarsus with a corresponding deviation of the tarsometatarsus, occurred more frequently in carcasses condemned for cellulitis, although in one study, the association was weak (10, 51). Valgus-varus deformity is considered to be the most frequent cause of leg weakness and lameness in broiler chickens (43). However, the association between valgus-varus deformity and coliform cellulitis needs to be interpreted with caution because of potential confounding with sex and breed. Most valgus-varus deformity affects male birds, and the incidence of coliform cellulitis can vary with breed. Birds with valgus-varus leg deformity spend more time lying on the floor (23), which results in greater contact exposure between the skin and the *E. coli* present in the litter. Also, prolonged resting by lame birds may result in skin damage as other birds tread on them (10).

Diagnosis

Cellulitis lesions are identified readily at processing, normally making it possible to use condemnation results to assess control strategies. However, an epidemiological study in Ontario has found that 30% of the variation in cellulitis prevalence was dependent on the slaughter plant (49). Therefore, the possibility of misclassification may exist, and should be considered in an investigation. Lesions should be cultured aseptically to determine the presence of *E. coli*. Currently, there is no *in vitro* method to distinguish cellulitis-type isolates from other types of the organism except by inoculation of scratches inflicted in the skin of older chickens (preferably greater than 3 weeks of age).

Prevention, Control, and Treatment

There is no treatment for coliform cellulitis, and eradication of the disease will not be possible because of the ubiquitous occurrence of *E. coli*. Recent advances in the development of immuno-

protective agents or immunomodulators suggest that a molecular approach to cellulitis control is possible, although not currently practical or economical (1). However, by carefully managing the environment and nutrition of the modern, fast-growing, heavy broiler, it is possible to substantially reduce the incidence and impact of the disease. A key aspect of any control strategy is its cost-benefit. Adequate monitoring to ensure implementation and compliance of control strategies will be needed to determine what is feasible. Interventions modifying the risk factors described previously can be envisioned, but the challenge will be to determine whether they are cost-effective. Following are some recommendations.

Time of Occurrence

Very early lesions consist mainly of serosanguineous fluid in contrast to the caseous lesions observed after 24 hours postinfection. When increased coliform cellulitis condemnations occur, it is important to determine the type of lesions (acute or chronic) found in the condemned birds. A high prevalence of acute lesions indicate that events occurring just prior to or during transportation should be investigated, especially if at least 10 hours exists between load-out and processing. In contrast, a majority of chronic lesions would indicate the need to focus on earlier events that occurred during the grow-out period.

Identify Risk Factors

Contrast problem flocks with flocks that did well during the same time period within the same company and determine the risk factors for each type of flock. Any management or environmental factors that affect the bird's resistance or contribute to skin scratches should be identified. Pay special attention to stocking density, feeder and waterer space (effective space—i.e., in some houses, the space is available, but the drinkers or feeders are not all functional), migration fencing, type of litter, quality of litter, and feed restriction and lighting programs. Any intervention must first focus on improving the environment of the birds. This includes good sanitation to reduce the bacterial load of the environment.

Monitor and Review Control Strategies

The best plan will fail if it is not fully implemented. Noncompliance is a key issue in health-related fields. Before judging the efficacy of a prevention measure, it is important to make sure that it was properly implemented.

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Chapter 19

Pasteurellosis and Other Respiratory Bacterial Infections

Introduction

John R. Glisson

A number of distinct respiratory diseases are caused by small gram-negative bacteria in commercial poultry. These diseases may have very similar clinical presentations. Many of the etiologic agents of bacterial respiratory diseases are classified as members of the family Pasteurellaceae but have in recent years been designated to new genera. Recent reclassifications included changing the name of *Pasteurella haemolytica* to *Gallibacterium anatis biovar haemolytica, Pasteurella gallinarum* to *Avibacterium gallinarum*, and *Haemophilus paragallinarum* to *Avibacterium paragallinarum*. The current taxonomy reflects advancements in techniques for determining genetic relatedness among bacteria. The new taxonomy is used in this text.

Four distinct diseases are included in this chapter: fowl cholera caused by *Pasteurella multocida*, *Riemerella anatipestifer* infection, *Ornithobacterium rhinotracheale* infection, and bordetellosis. These diseases are grouped together because they are caused by organisms that are genotypically and phenotypically related and because they induce diseases in commercial poultry that may present in a clinically similar way. Other diseases of poultry caused by members of the family Pasteurellaceae, such as fowl coryza caused by *Avibacterium paragallinarum*, are presented elsewhere in this text because the disease produced by infection with these organisms presents distinctly differently from the diseases included in this chapter.

In diagnostic poultry medicine, definitive diagnosis of fowl cholera, *Riemerella anatipestifer* infection, *Ornithobacterium rhinotracheale* infection, and bordetellosis is dependent upon the isolation and identification of the causative organism. Several organisms, such as *Avibacterium gallinarum,* which are of less importance as disease agents, may be isolated and must be differentiated from the more important disease agents included in this chapter. A clinical diagnostic text will be helpful in this regard (1).

References

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Fowl Cholera

John R. Glisson, Charles L. Hofacre, and Jens P. Christensen

Introduction

Fowl cholera (FC) (avian cholera, avian pasteurellosis, or avian hemorrhagic septicemia) is a contagious disease affecting domesticated and wild birds. It usually appears as a septicemic disease associated with high morbidity and mortality, but chronic or benign conditions often occur. This disease is of historical importance because of its role in the early development of bacteriology and because it was one of four diseases the Veterinary Division of the United States Department of Agriculture (USDA) was created to investigate.

History

Several epornitics among fowl occurred in Europe during the latter half of the 18th century. The disease was studied in France by Chabert in 1782 and in 1836 by Mailet, who first used the term *fowl cholera*. Huppe in 1886 referred to "hemorrhagic septicemia," and Lignieres in 1900 used the term *avian pasteurellosis*. Benjamin in 1851 gave a good description of the disease and demonstrated that it could be spread by cohabitation. With this knowledge of the disease, he formulated procedures for its

The contributions of Dr. Richard Rimler to this and previous editions of this chapter and his tremendous contribution to the body of knowledge on this subject is respectfully acknowledged.

prevention. At about the same time, Renault, Reynal, and Delafond demonstrated its transmissibility to various species by inoculation. In 1877 and 1878, Perroncito of Italy and Semmer of Russia observed in tissues of affected birds a bacterium that had a rounded form and occurred singly or in pairs. In 1879, Toussaint isolated the bacterium and proved it was the sole cause of the disease (54).

Pasteur (131) isolated the organism and grew pure cultures in chicken broth. In further studies, Pasteur (132, 133) used the FC organism to perform his classic experiments in attenuation of bacteria for use in producing immunity. Salmon (159) appears to have been the first to study the disease in the United States. A good description of disease signs was reported, however, as early as 1867 in Iowa, where losses of chickens, turkeys, and geese had occurred (7).

Incidence and Distribution

Normal fowl cholera is more prevalent in late summer, fall, and winter. This seasonal occurrence is one of circumstance rather than lowered resistance, except that chickens become more susceptible as they reach maturity.

Etiology

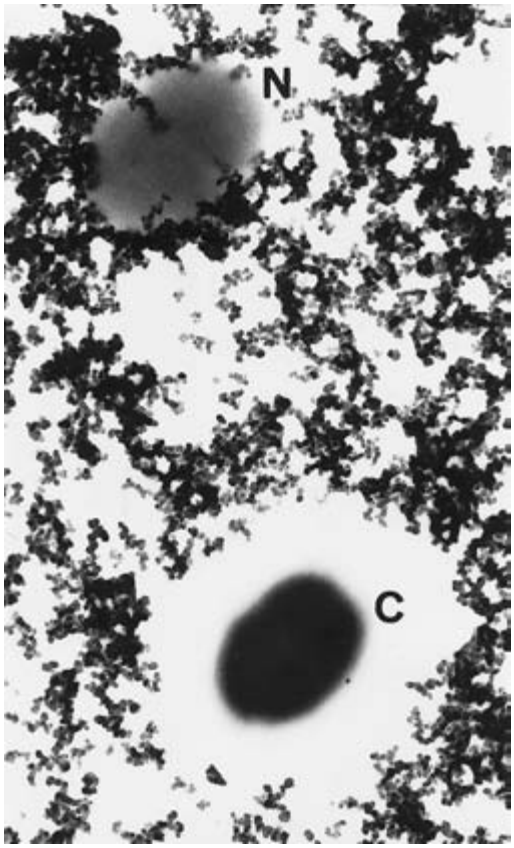
Classification

Pasteurella multocida is the causative agent of FC. When pronouncing *multocida*, the accent should be on the *ci* (19) rather than on the *to* as given in the 7th and 8th editions of *Bergey's Manual*. In the past, the bacterium has been given many names, including *Micrococcus gallicidus,* 1883; *M. cholerae gallinarum,* 1885; *Octopsis cholerae gallinarum,* 1885; *Bacterium cholerae gallinarum,* 1886; *Bacillus cholerae gallinarum,* 1886; *P. cholerae-gallinarum,* 1887; *Coccobacillus avicidus,* 1888; *P. avicida,* 1889; *Bacterium multicidum,* 1899; *P. avium,* 1903; *Bacillus avisepticus,* 1903; *Bacterium avisepticum,* 1903; *Bacterium avisepticus,* 1912; and *P. aviseptica,* 1920 (19, 22).

For a while, each isolate of *P. multocida* was named according to the animal from which it was isolated, such as *P. avicida* or *P. aviseptica, P. muricida* or *P. muriseptica.* In 1929, it was suggested that all isolates be referred to as *P. septica* (175). This name was used mainly in the United Kingdom and can be found in recent literature. *Pasteurella multocida,* proposed by Rosenbusch and Merchant (157), is now accepted as the official name in *Bergey's Manual* and is used exclusively throughout the world.

Morphology and Staining

P. multocida is a gram-negative, nonmotile, non-spore-forming rod occurring singly, in pairs, and occasionally as chains or filaments. It measures 0.2–0.4 × 0.6–2.5 mm but tends to become pleomorphic after repeated subculture. A capsule can be demonstrated in recently isolated cultures, using indirect methods of staining (Fig. 19.1). In tissues, blood, and recently isolated cultures, the organism stains bipolar (Fig. 19.2). Pili have been reported (51, 141).



19.1. Electron photomicrograph of *Pasteurella multocida*-encapsulated cell (C) and nonencapsulated cell (N) suspended in India ink. ×19,000.

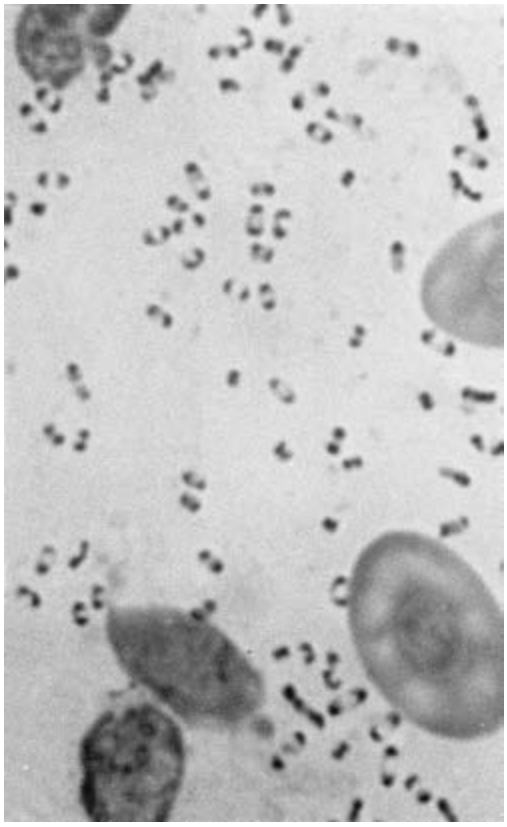
Growth Requirements

P. multocida grows aerobically or anaerobically. The optimal growth temperature is 37°C. The optimal pH range is 7.2–7.8, but growth can occur in the range 6.2–9.0, depending upon composition of the medium. In liquid media, maximum growth is obtained in 16–24 hours. The broth becomes cloudy, and in a few days, a sticky sediment collects. With some isolates, a flocculent precipitate occurs.

The bacterium will grow on meat infusion media; growth is enhanced when the medium is enriched with peptone, casein hydrolysate, or avian serum. Blood or serum from some animals inhibits growth of *P. multocida.* Inhibition is greatest from blood of horses, cattle, sheep, and goats; blood of chickens, ducks, swine, and water buffalo has little or no inhibitory action (158). Several selective media for isolation have been described (29, 30, 47, 99, 115, 165). Chemically defined media have been described by Jordan (91), Watko (172), Wessman and Wessman (174), and Flossmann *et al.* (45). Berkman (11) found that pantothenic acid and nicotinamide are essential for growth. Dextrose starch agar with 5% avian serum is an excellent medium for isolating and growing *P. multocida.*

Colonial Morphology and Related Properties

Colonial morphology observed with obliquely transmitted light is one of the most useful characteristics in the study of *P. multo-*

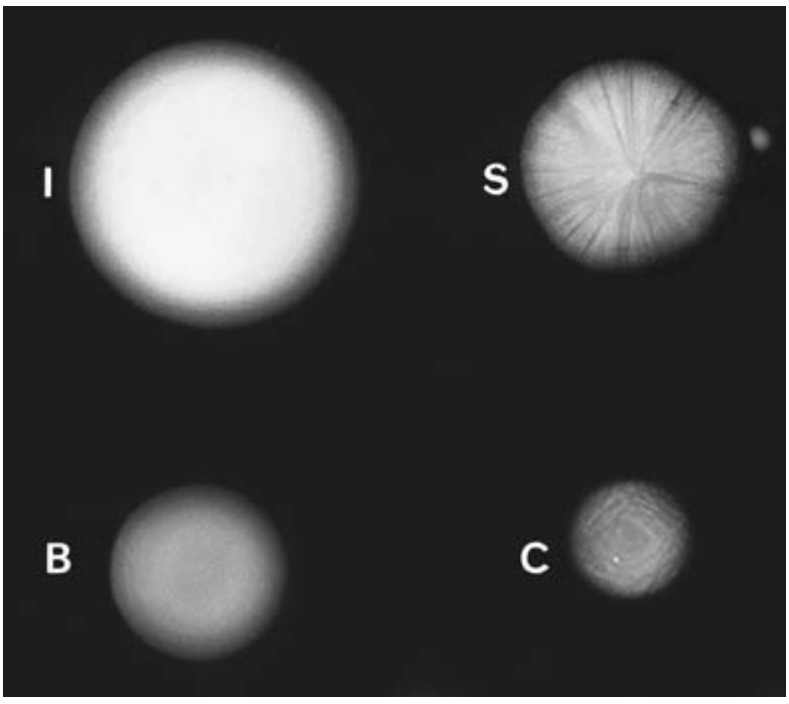


19.2. *Pasteurella multocida* in liver imprint from chicken with acute FC (note bipolarity). Wright's stain, ×2500.

cida. On primary isolation from birds with FC, colonies may be iridescent, sectored with various intensities of iridescence, or blue with little or no iridescence (Fig. 19.3). Iridescence is related to the presence of a capsule. The term *fluorescent* used to describe colonies in older literature should be considered synonymous with the term *iridescent*; the latter is the appropriate term.

The composition of the medium determines to a certain extent the degree and type of iridescence. Occasionally, an isolate produces blue colonies; when serum is added to the medium, sectored or iridescent colonies are sometimes produced. Examination of 18- to 24-hour colonies with a stereomicroscope using obliquely transmitted light (Fig. 19.4) is helpful when observing colonial morphology (76). Iridescent colonies on primary isolation from acute cases of FC are circular (2–3 mm), smooth, convex, translucent, glistening, and butyrous and show a tendency to coalesce. As the colony ages, it usually loses these distinguishing properties, becomes larger and viscous, and may adhere to the medium when picked with an inoculating needle. Blue colonies often isolated from birds with the chronic type of cholera or derived by dissociation of iridescent colonies are circular (1–2 mm), smooth, slightly convex or flat, translucent, butyrous, and discrete. The watery mucoid colonies produced by encapsulated strains from the respiratory tract of cattle, swine, sheep, rabbits, and humans are not iridescent but gray (70).

Anderson *et al.* (5) observed that a highly virulent isolate, which produced smooth colonies, later dissociated on subculture and produced rough colonies. Organisms from the smooth colonies were approximately 3–4 million times more virulent for pigeons than those from rough colonies. Hughes (81) studied the colonial morphology of 210 cultures from cases of FC and distinguished three types. The iridescent type was associated with



19.3. *Pasteurella multocida* 20-hour colonies on dextrose starch agar viewed with obliquely transmitted lighting (see Fig. 19.4). I. Iridescent. S. Sectored. B. Blue C. Rough. ×20.



19.4. Arrangement of stereomicroscope with obliquely transmitted light for evaluation of colonial morphology.

outbreaks of acute FC and was highly virulent. The blue type was of low virulence and occurred in flocks in which cholera was enzootic. The third type was intermediate in its properties of iridescence and virulence.

Heddleston *et al.* (71) reported that a virulent isolate of *P. multocida* of avian origin produced iridescent colonies that dissociated *in vitro* and produced blue colonies. Organisms from blue colonies also mutated and produced gray colonies, which have not been reported in primary cultures from birds. Cells from iridescent colonies occurred singly or in pairs, did not agglutinate in immune serum, were encapsulated, and were virulent for chickens, turkeys, rabbits, and mice when administered on mucous membranes of the upper air passages. Cells from blue colonies occurred singly or in pairs, were agglutinated by immune serum, were unencapsulated, and were avirulent when applied to mucous membranes of chickens and mice but were virulent for rabbits and slightly virulent for turkeys. Cells from gray colonies occurred only as chains and were unencapsulated and avirulent. Killed organisms from all three colonial forms induced immunity in chickens. Antigens extracted with hot saline from highly virulent encapsulated cells of iridescent colonies by Yaw and Kakavas (178) actively immunized chickens and mice, whereas less virulent unencapsulated cells from blue colonies immunized chickens more effectively than mice.

Physiologic Properties

The physiologic properties of *P. multocida* are used for identification. *P. multocida* does not produce gas but produces oxidase, catalase, peroxidase, and a characteristic odor. Unlike most gram-negative bacteria, it is sensitive to penicillin. The results of 29 other physiologic tests with 948 cultures of avian origin are shown in Table 19.1.

Significant differential characteristics are listed in Table 19.2.

Table 19.1. Physiologic properties of 948 *Pasteurella multocida* cultures of avian origin.

Test	% Positive
Arabinose	7.4
Dextrin	0.6
Dulcitol	2.6
Fructose	100.0
Galactose	99.8
Gelatin	0.0
Glucose	100.0
Glycerol	93.3
Hemolysis	0.0
Hydrogen sulfide	97.5
Indol	99.6
Inositol	0.0
Inulin	0.0
Lactose	1.6
Litmus milk	0.7
MacConkey agar	0.1
Maltose	0.0
Mannitol	99.5
Mannose	99.6
Motility	0.0
Nitrate reduction	100.0
Raffinose	2.7
Rhamnose	0.0
Salicin	0.0
Sorbitol	97.6
Sucrose	100.0
Trehalose	4.1
Urease	0.0
Xylose	77.4

Source: From Hacking, W. C. and J. R. Pettit. 1974. *Pasteurella hemolytica* in pullets and laying hens. *Avian Dis* 18: 483–486.

Table 19.2. Differential tests for *Pasteurella multocida*, *Gallibacterium anatis biovar haemolytica*, and *Avibacterium gallinarum*.

Test	*P. multocida*	*G. anatis biovar haemolytica*	*Av. gallinarum*
Hemolysis	−	+	−
MacConkey agar	−	+U	−
Indol	+	−	−
Motility	−	−	−
Gelatin	−	−	−
Catalase	+	+U	−
Oxidase	+	+	+
Urease	−	−	−
Glucose	+	+	+
Lactose	−U	+U	−
Sucrose	+	+	+
Maltose	−U	−U	+

Note: U = usually.

Resistance to Chemical and Physical Agents

P. multocida is destroyed easily by ordinary disinfectants, sunlight, drying, or heat, being killed within 15 minutes at 56°C and 10 minutes at 60°C. A 1% solution of formaldehyde, phenol, sodium hydroxide, betapropiolactone, or glutaraldehyde and a 0.1% solution of benzalkonium chloride killed within 5 minutes 4.4×10^8 organisms of *P. multocida*/ml suspended in 0.85% saline solution at 24°C.

Das (29) observed that cotton swabs saturated with blood from infected mice contained viable organisms after 118 hours but not after 166 hours (at which time the swabs were completely dry); films of blood on glass contained viable organisms after 24 but not 30 hours. Das also reported that infected blood sealed in glass tubes and held in a cold room contained viable organisms after 221 days. Skidmore (164) observed that the organism survived in dried turkey blood on glass for 8 but not 30 days at room temperature. In studies of environmental influence on the incidence of FC, Van Es and Olney (169) found the infection hazard had apparently disappeared from a poultry yard 2 weeks after occurrence of the last death and removal of birds.

Influence of temperature on viability and virulence of *P. multocida* was studied by Nobrega and Bueno (124), who observed that broth cultures stored in sealed tubes at an average room temperature of 17.6°C were still virulent after 2 years; at 2–4°C, they were nonviable after 1 year. With controlled experiments, Dimov (35) observed that *P. multocida* died rapidly in soils with moisture content of less than 40%. At a moisture content of 50% and temperature of 20°C, it survived for 5–6 days at pH 5.0, 15–100 days at pH 7.0, and 24–85 days at pH 8.0. A culture survived without loss of virulence for 113 days in soil with 50% moisture at 3°C and pH 7.15.

Cultures may be maintained without dissociation or loss of virulence in the lyophilized state or sealed in glass tubes and stored at 4°C or colder (173). Lyophilized cultures tested after 26 years were still virulent for chickens, and a culture sealed in a rubber-stoppered bottle containing beef infusion broth with 50% horse serum and held at room temperature was virulent after 26 years (63).

Subgrouping of P. multocida

Based upon DNA homology studies *P. multocida* has been divided into three subspecies namely *P. multocida* subspecies *multocida*, *P. multocida* subspecies *septica* and *P. multocida* subspecies *gallicida* (119). The subspecies can be differentiated by their physiologic properties (119). Important differential characteristics are listed in Table 19.3. By the use of full phenotypic characterization, Fegan *et al.* (43) were able to identify 10 biotypes among a strain collection including all three subspecies of *P. multocida* (43). All three subspecies of *P. multocida* have been isolated from outbreaks of fowl cholera (166, 77, 43). However, it appears that subspecies *multocida* is the most common subspecies isolated from chickens and turkeys and that only a minor percentage of isolations is made up by strains of subspecies *septica* (166, 43, 117). *P. multocida* subspecies *multocida* also seems to represent the predominant type of raptors and psittacines (113, 114) Subspecies *gallicida* is mainly associated with web-footed birds (77, 116, 52). The most recent taxonomic investigations of *P. multocida* include sequencing of 16S rRNA and housekeeping genes and indicate that subspecies *multocida* and *gallicida* belong to the same phylogenetic lineage and that subspecies *septica* makes up a separate lineage. Phenotypic criteria for separation of these two lineages, however, remain to be identified (15).

Table 19.3. Characters used for identification of subspecies of *Pasteurella multocida.*

	P. multocida		
Characters	**ssp. *multocida***	**ssp. *septica***	**ssp. *gallicida***
L(1) arabinose	v	−	+
D(2) arabinose	v	v	−
Dulcitol	−	−	+
D(2) sorbitol	+	−	+
L(2) fucose	v	v	−
Trehalose	v	+	−
Glucosidase (PNPG)	+	+	−

+: 90% or more of the strains positive within 1–2 days.
−: 90% or more of the strains negative.
v: variable.

Conventional subgrouping of *P. multocida* entails serological determination of capsular and somatic antigens. Specific capsule serogroup antigens are recognized using passive hemagglutination tests (24). Five capsular types (A, B, D, E, and F) are currently recognized (153). Carter (24) studied numerous isolates from various animals and found capsular types A and D were isolated from fowl and other animals. In a study of isolates representing a variety of avian hosts, Rhoades and Rimler (144) found organisms belonging to A, B, D, and F. Presumptive identification of capsular types A, D, and F can be determined by capsule depolymerization with specific mucopolysaccharidases (151). However, a highly specific multiplex capsular PCR assay has been developed which enables less laborious and more specific capsular typing than conventional tests. (168)

Somatic serotyping has been done by tube agglutination test (120) and gel diffusion precipitin methods (73). Comparative studies by Brogden and Packer (20) indicated that a serotype determination by one method did not correlate with a serotype determination by the other method. Often, cultures that represented a single somatic serotype in a particular test represented more than one serotype in the other test. Because of its simplicity, the gel diffusion precipitin test is used routinely in the United States, and its popularity is increasing throughout the world. The test uses antisera prepared in chickens and heat-stable antigens extracted from formalinized saline suspensions of the bacteria. The heat-stable antigens form lines of identity with lipopolysaccharide-protein complexes from culture supernatants (73). Somatic serotype specificity seems to be determined by the lipopolysaccharide component of a complex (149). Heddleston *et al.* (73) found there was good, though not absolute, correlation between

the gel precipitin test and the immune responese in chickens and turkeys. Rimler and Phillips (152) showed that lipopolysaccharide combined with carrier protein protected chickens against FC. To date, 16 somatic serotypes have been described (21). All of these somatic serotypes have been isolated from avian hosts. Correlation between subspecies and serovars of *P. multocida* obtained by traditional serotyping systems has not been demonstrated (16). During the years, somatic serotyping has provided useful information regarding the diversity of avian *P. multocida* strains, however, some particular serovars predominate in certain geographical regions making detailed conclusions concerning the epizootiology of *P. multocida* difficult. Thus, Snipes *et al.* (166) found that more than 60% of clinical *P. multocida* isolates from turkeys in California belonged to serovar 3,4 (161) and according to Gunawardana *et al.* serovar 1 dominates in Vietnam (56). In Australia, serovar 3 appears to dominate (16). In addition, phenotypic and genotypic variation within a given serovar has been demonstrated several times and underlines the limitations of serotyping as a method for strain differentiation (16, 23, 96, 176).

Other phenotypic methods of grouping strains of *P. multocida* have included phagetyping and Multi-Locus Enzyme Electrophoresis (MLEE). Phage sensitivity as a basis for grouping *P. multocida* has been investigated. Rifkind and Pickett (148) found that 84 of 118 isolates from various hosts were sensitive to one or more of 16 bacteriophages. Kirchner and Eisenstarck (97) examined 25 cultures of avian origin and found that 11 were lysogenic. They divided the 11 bacteriophages into five groups based on their host range, and into three groups based on plaque morphology. Karaivanov and Mraz (94) identified 87% of 77 cultures of *P. multocida* using one strain of bacteriophage. Saxena and Hoerlein (160) demonstrated lysogeny in 63 of 112 cultures from various hosts. One phage caused lysis of 8 different cultures; many were lysogenic for only 1 or 2 cultures. Gadberry and Miller (46) showed that 32 of 61 isolates were sensitive to 1 or more of 3 phages. Isolates of *Gallibacterium anatis, Avibacterium gallinarum, Actinobacillus ureae, [P.] pneumotropica*, and three species of the genus *Yersinia* were resistent to lysis. Results of these investigations demonstrated the possibility of a phage grouping system for *P. multocida*.

The analysis of isoenzymes, i.e. enzymes with the same functional activity but encoded by different alleles, has been used for many years in the study of eukaryotic population genetics (101), and has more recently been applied to prokaryotic organisms (161). Recently, Blackall *et al.* used MLEE to investigate the population stucture of Australian *P. multocida* isolates obtained from poultry (17). Although the 81 field isolates of *P. multocida* investigated were diverse, being divided into 56 electrophoretic types (ETs) the overall population structure was shown to form a clonal population in which little horizontal gene flow apparently had been occurring. No obvious correlation between serotype or subspecies and particular ETs was found.

Among phenotypically based methods used for grouping strains of *P. multocida* MLEE has provided the highest discriminatory power and has been suggested as a tool for elucidating the complex epizootiology of *P. multocida* infections (17). Data published on antibiotic resistance profiling do not seem to provide enough discriminatory power to allow its use for epidemiological investigations (126).

A number of nucleic acid based typing methods have been introduced for differentiation of avian strains of *P. multocida*. In favor of these methods, they do not depend on expression of phenotypic properties, all strains are typeable and the discriminatory power generally high (126). Bacterial genomes can be compared by electrophoretic separation of DNA fragments generated *in vitro* by digestion with restriction enzymes. To simplify the reading of results, the DNA fragments may be transferred to hybridization membranes and hybridized with a labelled hybridization probe (RFLP: Restriction Fragment Length Polymorphism), in which case the strain comparison is only based on restriction fragments that show homology to the probe. Strains that are of the same origin are anticipated to have the same genome and therefore both the same number and the same position of recognition sites for a restriction enzyme. Restriction enzyme analysis (REA) and RFLP of *P. multocida* have been used extensively to obtain knowledge about routes of transmission and about strain diversity in outbreak situations (23, 25, 113, 114, 166, 177). The restriction enzymes *Hpa*II and *Hha*I are most frequently used (26). Recently, also amplified fragment length polymorphism (AFLP) has proved useful for epidemiological purposes (41). This method is based on amplification of genomic fragments by the use of nonspecific primers following restriction endonuclease digestion and ligation of appropriate adaptors.In addition, RFLP analysis results have supported the population structure data on *P. multocida* obtained by MLEE (16). Pulsed Field Gel Electrophoresis (PFGE), using rare cutting restriction enzymes, allows the resolution of large DNA fragments reflecting restriction fragment length polymorphism throughout the entire genome. Gunawardana *et al.* (56) demonstrated a very high discriminatory power of PFGE using the technique on avian isolates of *P. multocida* originating from Australia and Vietnam.

In recent years, polymerase chain reaction (PCR) based methods have been used for differentiation of avian *P. multocida* isolates. Hopkins *et al.* succesfully used arbitrary primed PCR (AP-PCR) to distinguish the CU vaccine strain from fowl cholera outbreak strains (79). Repetitative extragenic palindromic sequence PCR (REP-PCR) has also been shown to be of value in identifying epidemiologically related and unrelated strains from Australia and Vietnam (56).

The use of plasmid profiling in subtyping of *P. multocida* has been of limited use due to a low number of plasmid carrying strains. Most investigations using this technique have shown that only approximately 20% of the isolates carry plasmids (25, 166).

Pathogenicity

Pathogenicity or virulence of *P. multocida* in relation to FC is complex and variable, depending on the strain, host species, and variations within the strain or host and conditions of contact between the two. The ability of *P. multocida* to invade and reproduce in the host is enhanced by the presence of a capsule (see Fig. 19.1) that surrounds the organism (104). Loss of ability of a virulent strain to produce the capsule results in loss of virulence

(71). Many isolates from cases of fowl cholera have large capsules but are of low virulence. Therefore, virulence is apparently related to some chemical substance associated with the capsule, rather than with its physical presence.

P. multocida usually enters tissues of birds through mucous membranes of the pharnyx or upper air passages, but it may also enter through the conjunctiva or cutaneous wounds. Hughes and Pritchett (82) were unable to infect chickens by placing a culture in a gelatin capsule and inserting it into the esophagus, but chickens were infected when the culture was dropped on the roof of the nasal cleft. Arsov (8) infected birds by mouth, using ^{35}P-labeled culture, and observed that the portal of infection was the mucous membrane of the mouth and pharnyx but not the esophagus, crop, or proventriculus. The eustachian tube was suggested by Olson and McCune (127) as the most likely route of infection, because the infection localizes in air spaces of the cranial bone, middle ear, and meninges.

Turkeys are much more susceptible than chickens to infection with *P. multocida,* and mature chickens are more susceptible than young ones (62). Hungerford (83) observed heavy losses in mature chickens, but no losses in birds up to 16 weeks of age in a case involving 90,000 birds. When testing infectivity of an isolate or susceptibility of a host, cohabitation is the most natural method of exposure. Unless the host is highly susceptible and the isolate highly invasive, however, results may be slow. Therefore, it is often advantageous to swab the nasal cleft with cotton saturated with the culture; if a more severe exposure is required, the culture can be injected parenterally.

Toxicity

A dried culture filtrate of *P. multocida* was first demonstrated to produce signs of toxicity in chickens by Pasteur (131). Salmon (159) repeated this work and described signs resulting from toxicity similar to those observed in cases of acute FC. Kyaw (100), using the developing chick embryo in the study of pathogenesis, suggested that a toxin was produced *in vivo* by *P. multocida.* Rhoades (143) observed severe general passive hyperemia in chickens that died from acute FC. This lesion was considered to be indicative of shock and was attributed to the action of endotoxin.

Endotoxins

Endotoxins are produced by all *P. multocida,* both virulent and nonvirulent. They may contribute to virulence; however invasion and multiplication of a strain are necessary for the production of sufficient quantities of endotoxin *in vivo* to contribute to pathologic processes.

Pirosky (137) obtained an endotoxin from *P. multocida* of avian origin by the trichloroacetic acid extraction procedure of Boivin. Heddleston and Rebers (67) demonstrated that a loosely bound endotoxin could be washed from *P. multocida* with cold formalinized saline solution. This endotoxin was a nitrogen-containing phosphorylated lipopolysaccharide, readily inactivated under mild acid conditions. Signs of acute FC were induced in chickens by injection of fractional amounts of endotoxin. The LD_{50} for chicken embryos was 5.2 mg via the chorioallantoic membrane; the LD_{50} for mice was 194 mg via the peritoneal cavity. One dose of 1.9 mg injected intravenously killed five of six 19-day-old turkeys; the median death time was only 3 hours. The endotoxin was present in the vascular system of turkeys with FC and could be detected with the Limulus lysate test and antiserum in the gel diffusion precipitin test. The serologic specificity of the endotoxin was associated with the lipopolysaccharide. Free endotoxin induced active immunity.

Purified lipopolysaccharides of each of the Heddleston serotypes were prepared by Rimler *et al.* (154). The lipopolysaccharides were similar to those of other gram-negative bacteria. Week-old poults were relatively resistant to the lethal effects of purified lipopolysaccharides from two highly pathogenic FC strains of *P. multocida* (145). In poults, the lipopolysaccharides did not provoke a dermal Shwartzman reaction, and lethality was not enhanced by a liver-damaging substance, a histamine-releasing substance, or a surgical bursectomy.

Protein Toxins

Heat-labile protein toxins have been found in serogroup A and D strains isolated from different animal species. Nielsen *et al.* (123) found 6 of 10 turkey strains produced heat-labile protein toxins; the strains were not serotyped. Four serogroup D strains isolated from turkeys were found to contain a heat-labile toxin (146). Sonicated suspensions of these strains produced necrotic lesions in turkey skin and were lethal to poults. Antiserum prepared against the heat-labile toxin from a swine strain neutralized the ability of the avian strain sonicated material to produce skin necrosis (147). Baba and Bito (9) chemically purified a protein toxin from an avian strain.

Pathogenesis and Epidemiology

Natural and Experimental Hosts

Most reported outbreaks of FC affected chickens, turkeys, ducks, or geese. However, this disease also affects other types of poultry, game birds raised in captivity, companion birds, birds in zoos, and wild birds. The wide range of avian hosts in which FC has been reported suggests that all types of birds are susceptible.

Among types of poultry, turkeys are most affected. Most or all in an infected flock may die within a few days. The disease usually occurs in young mature turkeys, but all ages are highly susceptible. Under experimental conditions, 90–100% of mature turkeys may die within 48 hours when exposed to a highly virulent strain of *P. multocida* by swabbing the palatine cleft or by contact with infected birds.

The disease in turkeys was first reported in detail by DeVolt and Davis (34), who described an outbreak in a flock of 175 turkeys in Maryland, where the mortality was 17%. Alberts and Graham (2) described outbreaks in four flocks of turkeys in which mortality was 17–68%. They emphasized that environmental stressors such as changes in climate, nutrition, injury, and excitement may have influenced the incidence and course of the disease.

Death losses from FC in chickens usually occur in laying flocks, because birds of this age are more susceptible than younger chick-

ens. Chickens less than 16 weeks of age generally are quite resistant. Fowl cholera in young chickens usually is caused by serotype 1 and often occurs in conjunction with some other malady. Recent outbreaks of FC in six flocks of 20–46-day-old broilers, however, resulted from infections with serotypes 3; 1,3; and 3,4. Experimental challenge of 5-week-old broilers with two representative strains (serotypes 3 and 1,3) resulted in mortality and lameness. In naturally infected chickens, mortality usually ranges from 0–20%, but greater losses have been reported. Reduced egg production and persistent localized infection often occur. Chickens are more susceptible to FC after withdrawal of feed and water or after an abrupt change of diet (18). Heat or rough treatment on a shaking machine increased the incidence in chickens exposed experimentally (92, 93).

Under experimental conditions, 90–100% of mature chickens exposed by swabbing the palatine cleft may die within 24–48 hours, depending on the strain of *P. multocida* used, but only 10–20% usually die within a 2-week period when exposed by contact with infected birds. Pritchett *et al.* (139) observed mortality of 35–45% in three houses of pullets. In one house, 45% of the birds died within 4 weeks. In a flock of 45 birds that had survived an acute outbreak the previous year, no losses were observed, but the number of birds with localized lesions increased during winter. In South Carolina and adjoining areas, FC existed mainly as a persistent, subacute chronic disease that clinically resembles avian monocytosis (13).

Domestic geese and ducks are also highly susceptible to FC. Curtice (28) reported the disease in geese in Rhode Island, where about 3200 of a flock of 4000 died in a short period. Van Es and Olney (169) recognized the marked susceptibility of geese to FC, in using them to test for persistence of viable organisms in lots after removal of infected chickens. Fowl cholera in ducks has been a serious problem on Long Island, where it was diagnosed on 32 of 68 commercial duck farms. Losses usually occur in ducks greater than 4 weeks of age, and mortality may reach 50% (40).

Birds of prey, waterfowl, and other birds kept in zoologic gardens occasionally succumb to infection; *P. multocida* has been isolated from more than 50 species of feral birds. During a $2^1/_2$-year survey, Faddoul *et al.* (42) isolated *P. multocida* from 13 (seven species) of 248 feral birds submitted to the diagnostic laboratory. Jaksic *et al.* (89) described an acute epornitic among pheasants, in which 1700 died. An outbreak in the San Francisco Bay area was reported to have been responsible for an estimated loss of 40,000 waterfowl (156). Gershman *et al.* (48) observed a serious outbreak among eider ducks (*Somateria mollissima*) in their nesting area 6 miles off the coast of Maine, where more than 200 birds died and more than 100 nests were lost. More than 60,000 waterfowl died of FC during the winter of 1956–1957 at the Muleshoe National Wildlife Refuge in Texas (90). Rosen (155) reported that there are two areas in the United States where fowl cholera is enzootic in waterfowl: the Muleshoe National Wildlife Refuge and the north central area of California. Both locations have had periodic outbreaks since 1944.

P. multocida from birds with FC usually will kill rabbits and mice, but other mammals are resistant to infection. According to Heddleston and Watko (69), rabbits, mice, pigeons, and sparrows died of acute septicemia when exposed intranasally to an isolate of *P. multocida* from an acute case of FC; rats, ferrets, guinea pigs, a sheep, a pig, and a calf did not show any clinical response to the same organism. One of 5 rats, 1 of 2 mink, and 11 of 19 mice fed viscera of infected chickens developed nasal infection, pneumonia, and fatal septicemia, respectively. A calf died of acute septicemia less than 18 hours after intramuscular (IM) exposure. Guinea pigs exposed by IM inoculation developed necrosis at the inoculation site; those exposed intraperitoneally usually died.

Horses, cattle, sheep, pigs, dogs, and cats are refractory to oral inoculation, and subcutaneous (SC) inoculation results in localized abscesses. All of these animals, however, may succumb to intravenous inoculation.

Transmission, Carriers, and Vectors

How FC is introduced into a flock is often impossible to determine. Chronically infected birds are considered to be a major source of infection. The only limit to the duration of the chronic carrier state is the lifespan of the infected bird. Free-flying birds having contact with poultry may be a source of FC organisms. Transmission of the organism through the egg seldom, if ever, occurs. A study of more than 2000 fresh and embryonated eggs from chickens infected with chronic FC yielded no evidence that *P. multocida* was transmitted through the egg (163).

Pritchett *et al.* (139, 140) and Pritchett and Hughes (138) examined three infected commercial flocks of white leghorns for *P. multocida* and found that many birds harbored the organism in nasal clefts. Presence of the bacterium was related to severity of upper respiratory infection in the flocks. They concluded that the enzootic focus of infection was healthy nasal carriers. These studies, as well as those of Van Es and Olney (169) and Hall *et al.* (58), proved that survivors of an epornitic of FC may be reservoirs of infection. Dorsey and Harshfield (39) reported a higher incidence of FC during late summer and fall in South Dakota. Carrier birds among the older flock, held over for a second year, provided a reservoir of infection for young susceptible pullets housed with them.

Most species of farm animals may be carriers of *P. multocida.* Generally, these organisms, except for those from swine and possibly those from cats, are avirulent for fowl. Iliev *et al.* (85) isolated *P. multocida* from tonsils of 34 of 75 slaughtered cattle, 14 of 27 sheep, and 102 of 162 pigs. Isolates from cattle and sheep were not pathogenic for fowl, but all 18 isolates from pigs in areas where FC was common were highly pathogenic for fowl. Only 2 of 47 isolates from pigs in areas having low incidence of FC were pathogenic. Iliev *et al.* (86) also reported that healthy pigs that were carriers of *P. multocida* transmitted infection to fowl in the same enclosure. Two isolates, serotypes 1:A and 5:A, from lungs of pigs with pneumonia, were studied by Murata *et al.* (118). Serotype 5:A was highly virulent for chickens, and serotype 1:A was avirulent. They found no cross-immunity in chickens between the two serotypes.

Gregg *et al.* (55) isolated two cultures from raccoons that were pathogenic for turkeys. They suggested that raccoons are a reser-

voir of *P. multocida*, and the organisms may be transmitted to turkeys via the raccoon bite. Contaminated crates, feed bags, or any equipment used previously for poultry may serve in introducing FC into a flock. Organisms are disseminated throughout the carcasses of birds that die of acute FC and may serve as an infection source, especially because fowl tend to consume such carcasses. Hendrickson and Hilbert (75) were able to isolate *P. multocida* from the blood of a naturally infected chicken for 49 days preceding death. They noticed a rapid increase in the number of organisms immediately preceding and following death and that the organisms remained viable 2 months at 5–10°C. Serdyuk and Tsimokh (162) demonstrated experimentally that sparrows, pigeons, and rats could become infected with *P. multocida* when exposed to chickens with FC and that they in turn could infect susceptible chickens. Sparrows and pigeons carried organisms without showing clinical signs, but 10% of infected rats developed acute pasteurellosis.

The possibility that insects may serve as vectors of FC has been investigated. Skidmore (164) experimentally transmitted FC to turkeys by feeding them flies that had previously fed on infected blood. He pointed out that under natural conditions, ingestion of flies might be a means of introducing the disease into a flock. Transmission by flies, however, is probably not common, as indicated by studies of Van Es and Olney (169). Although FC was maintained in two lots of chickens during the height of the fly season, no spread of the disease occurred to adjoining lots separated only by poultry netting. Iovcev (88) observed that larvae, nymphs, and adult ticks (*Argas persicus*) contained *P. multocida* after feeding on infected hens. Petrov (135) demonstrated that the red mite (*Dermanyssus gallinae*) became infected with *P. multocida* after feeding on infected birds, but the mite did not transmit the organism.

Heddleston and Wessman (70) showed that 27 cultures of *P. multocida* from the upper respiratory tract of humans were not pathogenic for turkeys. Humans can become infected, however, and may infect poultry via excretion from the nose or mouth.

Dissemination of *P. multocida* within a flock is primarily by excretions from the mouth (Fig. 19.5), nose, and conjunctiva of diseased birds that contaminate their environment, particularly feed and water. Feces very seldom contain viable *P. multocida*; however, Reis (142) found the organism in feces from 1 of 9 birds just before death. In the remaining 8 birds, the organisms were isolated only in feces collected from the cloacae of dead birds. Iliev *et al.* (87) demonstrated that *P. multocida* labeled with ^{32}P was inactivated in the proventriculus, and feces contained no viable *P. multocida.* Turkeys drinking from the same water trough with those experimentally infected with *P. multocida* developed FC (129).



19.5. Acute FC; mucous excretion from the mouth contains large numbers of *Pasteurella multocida* that can contaminate feed and water.

Signs of Infection

Acute

Signs of infection in acute FC are often present for only a few hours before death (see Fig. 19.5). Unless infected birds are observed during this period, death may be the first indication of disease. Signs that often occur are fever, anorexia, ruffled feathers, mucous discharge from the mouth, diarrhea, and increased respiratory rate. Cyanosis often occurs immediately prior to death and is most evident in unfeathered areas of the head, such as comb and wattles. Fecal material associated with the diarrhea is initially watery and whitish in color but later becomes greenish and contains mucus. Birds that survive the initial acute septicemic stage may later succumb to the debilitating effects of emaciation and dehydration, may become chronically infected, or may recover.

Chronic

Chronic FC may follow an acute stage of the disease or result from infection with organisms of low virulence. Signs generally are related to localized infections. Wattles (Fig. 19.6), sinuses, leg or wing joints, foot pads, and sternal bursae often become swollen. Exudative conjunctival (Fig. 19.7) and pharyngeal lesions may be observed, and torticollis (Fig. 19.8) sometimes occurs. Tracheal rales and dyspnea may result from respiratory tract infections. In the past, the term *roup* was used to indicate a condition in which signs were associated with chronic infections of cephalic mucous membranes. The term was not limited to FC, but included other diseases as well. Chronically infected birds may succumb, remain infected for long periods, or recover.

Gross and Microscopic Lesions

Lesions of FC are not constant but vary in type and severity. The greatest variation is related to the course of the disease, whether



19.6. Chronic FC; swollen wattle resulting from localized infection.



19.7. Chronic FC; serous inflammation of conjunctiva.



19.8. Chronic FC; torticollis resulting from meningeal infection.

acute or chronic. Although it is convenient for descriptive purposes to refer to either acute or chronic FC, it is sometimes difficult to categorize the disease in this manner. Signs of infection and lesions that occur may be intermediate to those described for acute and chronic forms.

Acute

When the course of the disease is acute, most of the postmortem lesions are associated with vascular disturbances. General hyperemia usually occurs, is most evident in veins of the abdominal viscera, and may be quite pronounced in small vessels of the duodenal mucosa (Fig. 19.9). Large numbers of bacteria usually can be observed microscopically in the hyperemic vessels. Petechial and ecchymotic hemorrhages are frequently found and may be widely distributed. Subepicardial (Fig. 19.10A) and subserosal hemorrhages are common, as are hemorrhages in the lung, abdominal fat, and intestinal mucosa. Increased amounts of pericardial and peritoneal fluid frequently occur. Disseminated intravascular clotting or fibrinous thrombosis has been observed in chickens and ducks that died from acute experimentally induced FC (84, 130).

Livers of acutely affected birds may be swollen and usually



19.9. Acute FC; hyperemia of chicken duodenum.

contain multiple small focal areas of coagulative necrosis (Fig. 19.10B) and heterophilic infiltration (Fig. 19.11). Some of the less virulent *P. multocida* do not produce necrotic foci in the liver. Heterophilic infiltration also occurs in lungs and certain other parenchymatous organs (143). Lungs of turkeys are affected more severely than those of chickens, with pneumonia being a common sequela. Large amounts of viscid mucus may be observed in the digestive tract, particularly in the pharynx, crop, and intestine.

Ovaries of laying hens are commonly affected. Mature follicles often appear flaccid; thecal blood vessels, which are usually easily observed, are less evident (Fig. 19.10E). Yolk material from ruptured follicles may be found in the peritoneal cavity. Immature follicles and ovarian stroma are often hyperemic.

Chronic

Chronic FC is characterized by localized infections, in contrast to the septicemic nature of the acute disease. These generally become suppurative and may be widely distributed anatomically. They often occur in the respiratory tract and may involve any part, including sinuses and pneumatic bones (Fig. 19.12). Pneumonia (Fig. 19.10C, D) is an especially common lesion in turkeys. Infections of the conjunctiva and adjacent tissues occur (see Fig. 19.7), and facial edema may be observed. Localized infections may also involve the hock joints (Fig. 19.10F), foot pads, peritoneal cavity, and oviduct.

Chronic localized infections can involve the middle ear and cranial bones and have been reported to result in torticollis. In turkeys, torticollis and eventual death can be associated with infections of the cranial bones, middle ear, and meninges. In a study of naturally infected turkeys exhibiting torticollis, Olson (125) described lesions at these sites. The outstanding gross lesion was yellowish caseous exudate in air spaces of the calvarial bones. Heterophilic infiltration and fibrin were consistently observed in the air spaces, middle ear, and meninges. Multinuclear giant cells were often associated with necrotic masses of heterophils in air spaces. Similar lesions were found in experimentally exposed turkeys (127). Localized meningeal infections (Fig. 19.13), without involvement of cranial bones or the middle ear, have been observed in turkeys exhibiting torticollis, as have cerebellar infections (44).

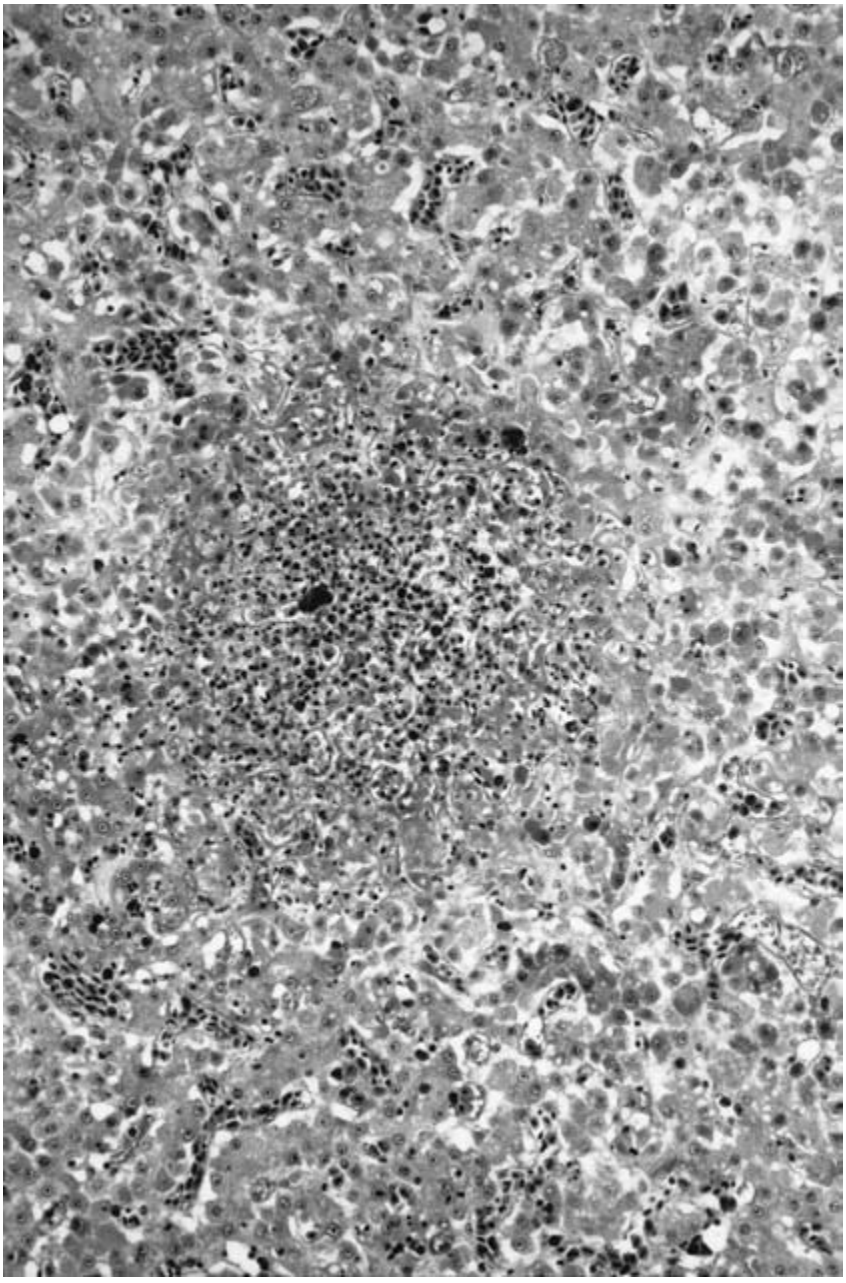
Immunity

Pasteur (133) used an avirulent culture attenuated by prolonged growth on artificial medium and produced immunity that protected fowl against subsequent exposure. In field use, his method did not prove practical because uniform attenuation could not be obtained, and heavy losses sometimes occurred in vaccinated flocks.

Since Pasteur's classic work, numerous attempts have been made to produce efficient vaccines against FC, but results have not been consistent. There can be little doubt, however, that a substantial, but not absolute, immunity can be induced in fowl by using killed *P. multocida* vaccines under controlled conditions (10, 64). Killed *P. multocida* vaccines usually are prepared by growing selected immunogenic strains on a suitable medium and suspending them in formalinized saline solution. The killed organisms usually are incorporated with an adjuvant and injected subcutaneously.

Under field conditions, losses from FC sometimes occur in vaccinated flocks. This failure may be due to improperly prepared or administered vaccine or immune impaired birds. Heddleston and Reisinger (68) demonstrated that stress caused by changing the social or peck order of vaccinated males, as well as fowlpox infection in chickens at time of vaccination and exposure, significantly reduced the efficacy of vaccination. In experimental studies (136), the manifestation of acquired resistance was impaired in turkeys vaccinated against *P. multocida* while receiving aflatoxin in their feed. It was also observed that an isolate of *P. multocida* recovered from an FC outbreak in previously vaccinated turkeys differed serologically from the culture used in preparing vaccine (72).

In experimental studies, Heddleston and Rebers (66) showed that bacterins prepared with tissues from infected turkeys or live *P. multocida* administered in drinking water will induce immunity in turkeys against a different immunogenic type. A bacterin prepared with bacteria grown on conventional agar media did not induce cross-immunity. These studies indicate that *P. multocida* produces a wider spectrum of immunogens *in vivo* than *in vitro*. Rimler (150) showed that turkeys vaccinated with *in vivo*-grown *P. multocida* and challenged with the homologous strain produced serum that passively immunized poults against five different serotypes. Bierer and others at Clemson University stimu-

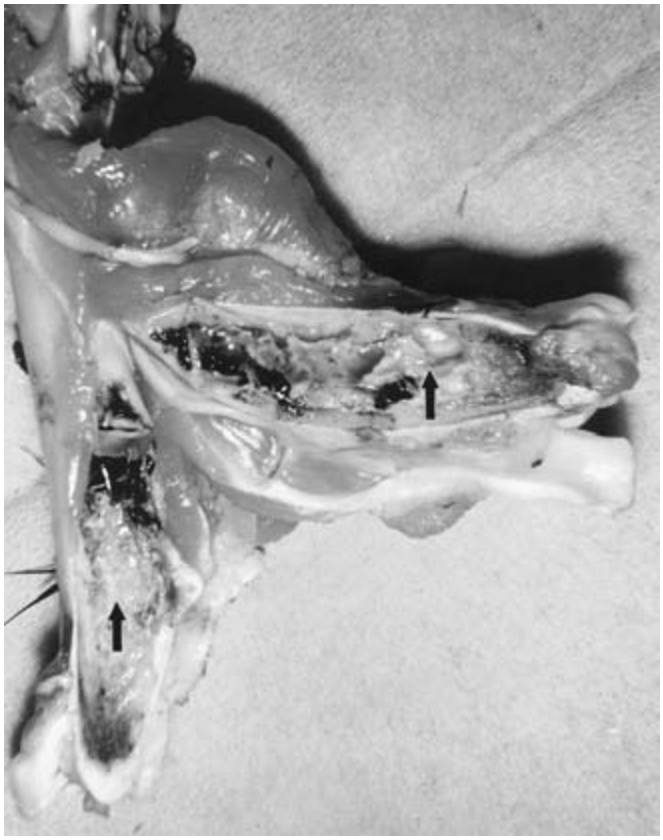


19.11. Acute FC; coagulative necrosis and heterophilic infiltration in turkey liver. H & E, ×600.

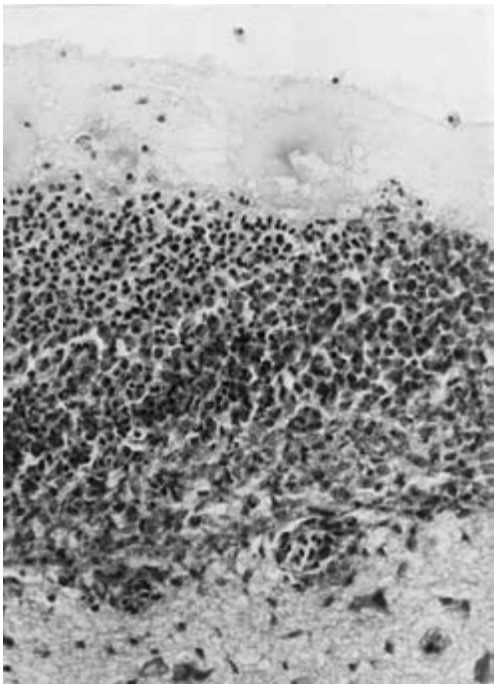
lated renewed interest in live FC vaccine administered in drinking water. Bierer and Derieux (14) demonstrated good immunity in 14-week-old turkeys that were given a live culture of *P. multocida* (CU strain, previously CS-148) in drinking water 2 weeks before challenge exposure. The vaccine, however, killed 4.2% of 120 turkeys. Best results were obtained by inoculating 8-week-old turkeys with a killed bacterin and then administering the live vaccine 2 weeks later; the live vaccine killed only 2.5% of 120 turkeys. Derieux and Bierer (33) stated that good immunity may be obtained in 6-week-old turkeys by administering 2 doses of vaccine in drinking water on the same day and repeating the vaccination 4 weeks later. No data were given, however, as to duration of immunity or number of turkeys killed by vaccination. The CU strain administered in drinking water was immunologically less effective in chickens than in turkeys. It was more effective in chickens by wing-web or SC inoculation than in drinking water (32). Live vaccines are commercially available for oral administration to turkeys and parenteral administration to chickens.

Maheswaran *et al.* (103) also induced immunity in turkeys with live vaccines via drinking water; they suggested that the vaccine induced localized, but not systemic, protection. In other studies, Heddleston *et al.* (74) showed that serum from birds vaccinated via drinking water would induce passive immunity in chicks and turkeys.

Passive immunity for prevention of FC was studied in 1892 by Kitt, who used immune horse serum. This method was used frequently, but because of the short duration of passive immunity, it is presently used little if at all. Bolin and Eveleth (18) reported



19.12. Chronic FC; caseous exudate (arrows) in turkey humerus.



19.13. Chronic FC; fibrinoheterophilic meningitis in turkey. H & E, ×400.

that *P. multocida* antiserum prepared in chickens gave maximum protection 16–24 hours after injection; protection began to decline after 48 hours and had disappeared after 192 hours.

Diagnosis

A presumptive diagnosis of FC may be made from clinical observations, necropsy findings, or isolation of *P. multocida*; a conclusive diagnosis should be based on all three. Signs and lesions of the disease were described previously.

Isolation and Identification

P. multocida can be isolated readily from viscera of birds that die of acute FC and usually from lesions of chronic cases; it is less likely to be isolated from dehydrated, emaciated survivors of an acute outbreak. A tentative diagnosis of acute FC can be made by demonstrating bipolar organisms in liver imprints (see Fig. 19.2) using Wright's stain. Immunofluorescent microscopy can be used to identify *P. multocida* in tissue or exudate (105).

Bone marrow, heart blood, liver, meninges, or localized lesions are preferred for culturing. To isolate *P. multocida,* sear the tissue or exudate with a spatula and obtain a specimen by inserting a sterile cotton swab or wire loop through the seared surface. If birds are living, squeeze mucus from the nostril or insert a cotton swab into the nasal cleft. Transfer the specimen to peptone broth and streak on dextrose starch agar containing 5% chicken serum or other suitable media. Specimens may also be streaked on MacConkey and blood agar media to aid in identification.

Colonies characteristic of *P. multocida* (described under Etiology) are transferred to dextrose starch agar slants incubated 18–24 hours. Tubes of phenol red broth base containing 1% glucose, lactose, sucrose, mannitol, and maltose, respectively, are then inoculated with growth from the slant. Fermentation of glucose, sucrose, and mannitol without gas is characteristic of *P. multocida.* Lactose is usually not fermented, but some avian isolates will ferment it. Inoculate 2% tryptose in 0.85% saline solution, incubate 24 hours at 37°C, and test for indole (Kovac's test). Indole is almost always produced by *P. multocida.* There should be no hemolysis of blood and no growth on MacConkey agar (Table 19.2).

Inoculation of animals may be used as an aid in isolating *P. multocida* from contaminated materials. Rabbits, hamsters, or mice are inoculated subcutaneously or intraperitoneally with 0.2 ml exudate or minced tissue. If *P. multocida* is present, the animal usually dies within 24–48 hours, and the organism can be isolated in pure culture from heart, blood, or liver.

Serologic diagnosis of FC by rapid whole-blood agglutination, serum plate agglutination, agar diffusion tests, or ELISA has limited value in chronic cholera and no value with the acute form of the disease.

Differential Diagnosis

Avibacterium gallinarum and *Gallibacterium anatis biovar haemolytica* are two closely related bacteria that may be isolated

from diseased poultry and incorrectly identified as *P. multocida* (65). *Avibacterium gallinarum* was first described by Hall *et al.* (58), who isolated it along with *P. multocida* from chickens with other maladies characterized by inflammation of the upper respiratory tract. The gel diffusion precipitin test shows a common antigen between *Avibacterium gallinarum* and *P. multocida.* Clark and Godfrey (27) found *Avibacterium gallinarum* associated with a respiratory disease complex of chickens in southern California. Gilchrist (49), in a survey of avian respiratory diseases in New South Wales, reported finding *Avibacterium gallinarum, Gallibacterium anatis biovar haemolytica,* and *P. multocida.* Harbourne (60) isolated *Gallibacterium anatis biovar haemolytica* on four occasions from livers of young chickens and turkeys. *Gallibacterium anatis biovar haemolytica* was isolated from young chickens with salpingitis, which was often accompanied by nasal catarrh, helminth infection, or leukosis; the organism was also isolated from the lungs of fowl with chronic respiratory disease and infectious bronchitis (122). Matthes *et al.* (106) isolated *Gallibacterium anatis biovar haemolytica* from chickens with a septicemia. Chloramphenicol was effective in treatment. Hacking and Pettit (57) reported on 8 cases of *Gallibacterium anatis biovar haemolytica* in pullets and laying hens: 5 cases involved egg production, with some birds showing peritonitis or salpingitis; 3 cases involved mortality; some birds had enteritis, enteritis, and hepatitis, or respiratory infection. In most cases, *Gallibacterium anatis biovar haemolytica* was thought to be a secondary pathogen.

Differential characteristics of *P. multocida, Avibacterium gallinarum,* and *Gallibacterium anatis biovar haemolytica* that may be isolated from poultry are listed in Table 19.2.

Treatment

Antibacterial chemotherapy has been used extensively in the treatment of FC with varying success, depending to a large extent on the promptness of treatment and drug used. Sensitivity testing is often advantageous, because strains of *P. multocida* vary in susceptibility to chemotherapeutic agents (37, 171), and resistance to treatment may develop, especially during prolonged use of these agents.

Sulfonamides

Several of the sulfonamides have been employed both experimentally and in naturally occurring outbreaks. The main disadvantages of the sulfonamides are their bacteriostatic instead of bactericidal action, inability to cure localized abscesses, and their toxic effect on birds. Kiser *et al.* (98) reported 63–85% reduction in mortality from experimentally produced FC compared with untreated controls when using sulfamethazine and sodium sulfamethazine. In naturally occurring outbreaks, mortality was reduced 45–75%. Favorable results were obtained with 0.5–1% of the drug in food, or 0.1% in drinking water.

Alberts and Graham (3) employed 0.5% sulfamerazine in mash feed for 5 days in a field outbreak of FC in turkeys. Mortality was 1.9% in the treated group compared with 50% in untreated birds. Fowl cholera recurred four times after cessation of treatment, and each time losses were arrested after turkeys were again given the sulfamerazine-mash mixture. In experimental infection in turkeys, sodium sulfamerazine at oral dosage rates of 143 and 107.25 mg/kg body weight effectively reduced mortality. In chickens, 0.2% sodium sulfamerazine in drinking water or 0.4% sulfamerazine in mash checked mortality in an established outbreak 2 days after treatment was started (1). Sulfaquinoxaline in amounts of 0.01–0.05% in drinking water was completely prophylactic in experimental FC when treatment was started 24 hours before birds were inoculated. Peterson (134) treated two naturally occurring outbreaks in turkey flocks successfully with 1:2000–1:4000 dilution of the drug in drinking water. He found sulfamethazine and sodium sulfamerazine also were markedly effective in reducing experimental FC; sulfadiazine, sulfathiazole, and sulfanilamide were much less so. Sulfaquinoxaline was used by Delaplane (31) at the rate of 0.1% or 0.05% in mash in prophylaxis of FC in chickens. Nelson (121) reported favorable results in controlling mortality in turkeys with a concentration of 0.025% sulfaquinoxaline in drinking water for 5–7 days; he stated also that its administration 1 day out of 4 usually controls later mortality and permits the grower to finish birds for market. Dorsey and Harshfield (39) confirmed the usefulness of several sulfonamide drugs in checking losses from FC if treatment is carried out in early stages of an outbreak. They also noted frequent recurrence of mortality after treatment was discontinued and unsatisfactory results of treatment after the disease had become chronic.

Sulfaethoxypyridazine was reported by Stuart *et al.* (167) to be effective in controlling FC in chickens and turkeys. Effectiveness of the drug was dependent in part on size of dose and on the duration and promptness of treatment. Sulfadimethoxine, used alone or potentiated with ormetoprim, was found to be safe, palatable, and effective against experimentally induced FC in chickens and turkeys (109, 110, 111, 112, 167). Anderson *et al.* (6) reported that sulfachloropyrazine administered in drinking water was effective in preventing mortality in experimentally exposed chickens.

Antibiotics

Streptomycin given IM in a dose of 150,000 mg prevented deaths in adult turkeys when administered before or at the time of inoculation of *P. multocida.* When treatment was delayed for 6–24 hours or dosage was reduced, chronic infection resulted (108). Penicillin, streptomycin, penicillin and streptomycin, and oxytetracycline (administered IM at the time of experimental exposure of chickens) all possessed activity as therapeutic agents (13). Chlortetracycline reduced losses in chicks about 80% when given at the rate of 40 mg/kg body weight IM 30 minutes after parenteral inoculation of the organism (102). Chicks that received mash containing 1 mg/g had 50% fewer losses than untreated controls. In an outbreak of FC in pheasants, however, Alberts and Graham (4) did not observe any beneficial results when 1 mg/g mash was fed. When chlortetracycline was given IM, a slight reduction in mortality was recorded. Novobiocin administered in feed or water reduced death losses in experimentally exposed turkeys (59). Chloramphenicol (20 mg/kg body

weight) in a single IM injection was effective in treating FC, but in flocks in which FC and fowl typhoid or fowlpox were concurrently present, chloramphenicol treatment was not successful (80). A chloramphenicol-dexamethasone-pyribenzamine combination was used successfully with vaccination in treatment of FC in breeding turkeys. Respiratory problems, which occurred 1 week after the initial outbreak, responded readily to IM administration of this drug combination (53). Water-soluble erythromycin at the rate of 1 lb/50 gal drinking water halted mortality in two flocks of Muscovy ducklings infected with *P. multocida* (61). Fluoroquinolones are used successfully to treat fowl cholera. *Pasteurella multocida* isolates from poultry are typically highly susceptible to fluoroquinolones (50).

Antibiotics used in rations at very low levels for promotion of growth, according to the experiments of Dorsey and Harshfield (39), did not significantly influence the course of FC infection in inoculated birds. At therapeutic levels, birds that received penicillin and streptomycin in feed died at about the same rate as controls. No deaths occurred in groups that received sulfaquinoxaline or sulfamerazine. These workers found oxytetracycline and chlortetracycline effective also in preventing mortality in experimental FC in a small flock of laying birds; mortality was 80% in an untreated group compared with 12% in a group receiving mash containing oxytetracycline at the level of 500 g/ton. In six naturally occurring outbreaks, oxytetracycline at this level in feed checked mortality, but losses returned in three flocks after withdrawal of the antibiotic.

Prevention and Control

Management Procedures

Prevention of FC can be effected by eliminating reservoirs of *P. multocida* or by preventing their access to poultry flocks. Good management practices, with emphasis on sanitation as prescribed by Zander, Bermudez, and Mallinson (see Chapter 1), are the best means of preventing FC. Unlike many bacterial diseases, FC is not a disease of the hatchery. Infection, therefore, occurs after birds are in the hands of the producer, and consideration must be given to the many ways that infection might be introduced into a flock.

The primary source of infection is usually sick birds or those that have recovered and still carry the causative organism. Only young birds should be introduced as new stock; they should be raised in a clean environment completely isolated from other birds. Isolation should be extended to housing. Unless separate houses can be provided for first- and second-year layer flocks, the older flock should be marketed in its entirety. Different species of birds should not be raised on the same premises. The danger of mixing birds from different flocks cannot be overemphasized. Farm animals (particularly pigs, dogs, and cats) should not have access to the poultry area. Water fountains should be self-cleaning, and feeders should be covered to prevent contamination as much as possible.

That *P. multocida* has been recovered from many species of free-flying birds warrants consideration of this source of infection to poultry, with measures taken to prevent their association with the flock. Raising turkeys in areas where FC is a serious problem may warrant their confinement in houses from which free-flying birds, rodents, and other animals can be excluded. If an outbreak of FC occurs, the flock should be quarantined and disposed of as soon as economically feasible. All housing and equipment should be cleaned and disinfected before repopulation.

Immunization

Vaccination should be considered in areas where FC is prevalent, but it should not be substituted for good sanitary practice. Commercially produced bacterins and live vaccines are available. Bacterins usually contain whole cells of serotypes 1, 3, and 4 emulsified in an oil adjuvant. Because a bacterin will not provide protection against a FC challenge from a serotype not contained in that bacterin, an autogenous whole-cell bacterin containing a locally isolated strain other than serotypes 1, 3, or 4 may be used (62). The choice of adjuvant for an autogenous vaccine can be water-in-oil emulsion or aluminum hydroxide (12). Autogenous bacterins using aluminum hydroxide as the adjuvant are useful for the vaccination of turkey breeder or broiler breeder flocks that are in lay because the water-in-oil emulsion, in combination with the whole bacterial cell, results in a significant tissue response by the bird. This response can result in significant declines in egg production. The negative effect on egg production is less with aluminum hydroxide adjuvant whole-cell FC bacterins. It has been well documented that aluminum hydroxide bacterins do not stimulate the immune response as well as water-in-oil bacterins (68, 107). Therefore, if an aluminum hydroxide bacterin is used, revaccination may be required to afford immunity to a flock for an entire laying cycle.

Three live vaccines available for use in the United States are CU (Clemson University), a strain of low virulence; M-9, a mutant of CU with very low virulence; and PM-1, a mutant of CU intermediate in virulence between CU and M-9. Vaccination of chickens and turkeys with these live *P. multocida* vaccines induces protection against heterologous serotype challenge. The use of live FC vaccines stimulates an effective immune response but has the disadvantage of potentially resulting in mortality in the vaccinated birds (14). If the mortality post-vaccination becomes excessive, it can be reduced by the administration of an antibiotic. This should be avoided, if possible, until at least 4 days post-vaccination when there will be at least partial immunity induced by the vaccine (128).

When considering the most appropriate vaccination program for FC, the following should be taken into consideration: prevalence of FC in the area, most prevalent serotypes of *P. multocida* in area, age of birds to be vaccinated, and the value of the birds to be vaccinated (i.e., breeder turkeys versus commercial turkeys or parent chicken breeders versus grandparent chicken breeders). There have been many successful vaccination protocols for chicken breeders against FC. Bacterins, live vaccines, or both are used, and usually two doses are given: the first at 8–10 weeks of age and the second at 18–20 weeks of age. Protection occurs only against serotypes contained in the bacterin and does not give solid immunity for an entire laying cycle. Some of the more commonly used vaccination programs consist of administering a live vaccine

in the wingweb at 10–12 weeks of age followed by either another live vaccine in the wingweb or a bacterin at 18–20 weeks. Vaccination with live vaccine provides protection against multiple serotypes, but the vaccine can cause chronic FC. The use of a bacterin at 10–12 weeks and a live vaccine at 18–20 weeks, just prior to movement to the laying house, gives protection against multiple serotypes and minimizes live vaccine induced chronic FC (78).

One of the most successful programs for vaccination of both breeder turkeys and commercial/or meat turkeys is the use of a live vaccine in the drinking water every 4 weeks beginning at 6–8 weeks of age and continuing for the life of the flock. Bacterins can also be used in breeder turkeys. They are vaccinated 2–5 times before the onset of egg production, with the first vaccination beginning at 6–8 weeks.

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Riemerella anatipestifer Infection

Tirath S. Sandhu

Introduction

Definition and Synonyms

Riemerella anatipestifer (RA) infection is a contagious disease of domestic ducks, geese, turkeys, and various other domestic and wild birds. It is also known as new duck disease, duck septicemia, anatipestifer syndrome, anatipestifer septicemia, and infectious serositis. In geese, RA infection has been called goose influenza or septicemia anserum exsudativa (40). It occurs as an acute or chronic septicemia characterized by fibrinous pericarditis, perihepatitis, airsacculitis, caseous salpingitis, and meningitis.

Economic Significance

R. anatipestifer infection is a major disease confronting the duck industry throughout the world. It accounts for significant economic losses due to high mortality, weight loss, condemnations, downgrading, and salvage. Prevention and control programs consisting of vaccination and treatment add to the cost.

Public Health Significance

The disease is of no public health significance.

History

R. anatipestifer infection was first described in 1932 in Pekin ducks from three farms on Long Island, New York (28). The report referred to a new disease, which became known in the area as "new duck disease." The disease started in 7- to 10-week-old ducks with about 10% mortality and later spread to younger ducklings of about 3 weeks of age. Six years later, the disease was observed in ducks from a commercial farm in Illinois and was reported as "duck septicemia" (20). The designation "infectious serositis" was given by Dougherty and coworkers (16) after a comprehensive pathologic study. The term *R. anatipestifer infection* was recommended by Leibovitz (39) to identify the disease specifically caused by *R. anatipestifer* and to differentiate it from other infections with similar pathology. A similar disease, septicemia anserum exsudativa, was described in geese by Riemer (55). The causative agent, *Pasteurella septicaemiae*, is identical to RA on the basis of reported characteristics (30, 69).

Etiology

Classification

The causative bacterium was isolated and characterized by Hendrickson and Hilbert (28), who called it *Pfeifferella anatipestifer.* Bruner and Fabricant (10) studied and compared its characteristics with those of *Brucella, Pasteurella, Moraxella, Actinobacillus,* and *Haemophilus.* They concluded that the organism had more in common with *Moraxella* sp. and suggested the name *Moraxella anatipestifer.* It was listed in the seventh edition of *Bergey's Manual of Determinative Bacteriology* as *Pasteurella anatipestifer* (8). Because of its uncertain taxonomic status, it was placed as species *incertae sedis* in the eighth (69) and ninth (43) editions of *Bergey's Manual of Systematic Bacteriology*. Comparison of its DNA base composition, DNA-DNA homology, and cellular fatty-acid profile indicated its exclusion from the genus *Moraxella* as well as *Pasteurella* (5, 43). Piechulla *et al.* (53) suggested the transfer of RA to the *Flavobacterium*/*Cytophaga* group on the basis of low but significant DNA binding and production of menaquinones and branched-chain fatty acids. Segers *et al.* (68) reported significant differences between RA and its close genotypic relatives *Flavobacterium* and *Weeksella.* They suggested placing this organism in a separate genus *Riemerella*, in honor of Riemer (55), who first described the disease "septicemia anserum exsudativa" in geese in 1904, and named it *Riemerella anatipestifer* on the basis of a DNA-ribosomal RNA hybridization analysis, its protein and fatty acid methylester (FAME) profiles, and its phenotypic characteristics such as lack of pigment production and presence of respiratory quinone "menaquinone 7."

R. anatipestifer-like organisms of taxon 1502 (32) isolated from ducks and geese were assigned to the genus *Coenonia* and named *Coenonia anatina* gen. nov., sp. nov. on the basis of phenotypic and genotypic characteristics and FAME profiles (74). *C. anatina* differed from RA by the absence of arginine dihydrolase and gelatinase and by the presence of hyaluronidase, chondroitin sulfatase activity, aesculin hydrolysis, and β-glucosaminidase activity.

Morphology and Staining

R. anatipestifer is a gram-negative, nonmotile, nonspore-forming rod occurring singly, in pairs, and occasionally in chains. The cells vary from 0.2–0.4 mm in width and 1 to 5 mm in length. Many cells stain bipolar with Wright's stain, and a capsule can be demonstrated in preparations with India ink.

Growth Requirements

The organism grows well on chocolate agar, blood agar, or trypticase soy agar. Growth of fastidious strains can be enhanced by the addition of 0.05% yeast extract and 5% newborn calf serum. Growth is more abundant with increased carbon dioxide (20). Hendrickson and Hilbert (28) described the organism as a strict aerobe on the basis of results obtained with the pyrogallic acid and sodium hydroxide procedure for removing oxygen. However, because carbon dioxide would also be depleted by reacting with the sodium hydroxide, neither oxygen nor carbon dioxide was available to the organism. Although some strains of RA grow at an incubation temperature of 45°C, no growth is observed at 4°C or 55°C (4); maximum growth usually occurs in 48–72 hours when incubated at 37°C in a candle jar that provides increased carbon dioxide and moisture, both of which favor growth.

Colony Morphology

Colonies on blood agar, when grown 24–48 hours at 37°C in a candle jar, are 1–2 mm in diameter, convex, entire, transparent, glistening, and butyrous. Some strains produce slimy growth. Colonies on clear media are iridescent when observed with obliquely transmitted light.

Biochemical Properties

Carbohydrates are not fermented by routine sugar fermentation tests. However, acid production has been detected in dextrin, glucose, maltose, inositol, trehalose, mannose, and fructose by growing in buffered single substrate medium (2, 4, 31). Gelatin is usually liquified, and litmus milk may slowly turn alkaline. Usually, indol and hydrogen sulfide are not produced; however, some strains are indol positive (32). Nitrate is not reduced to nitrite, and starch is not hydrolyzed. There is no growth on MacConkey agar and no hemolysis on blood agar. *R. anatipestifer* is oxidase- and catalase-positive; phosphatase is produced (22). It is negative for aesculin hydrolysis, hyaluronidase, and chondroitin sulfatase (32). Some strains produce urease and arginine dihydrolase.

R. anatipestifer is positive for acid and alkaline phosphatase; ester lipase C8 (APIZYME system); leucine-, valine-, and cystine-arylamidases; phosphoamidase; α-glucosidase; and esterase C4 and while negative for the following enzyme activities: α- and ß-galactosidases, ß-glucuronidase, ß-glucosidase, α-mannosidase, ß-glucosaminidase, lipase C14, fucosidase, and ornithine and lysine decarboxylases (53, 68).

Susceptibility to Chemical and Physical Agents

Most RA strains do not survive on solid media for more than 3–4 days at 37°C or room temperature; cultures in broth may be viable for 2–3 weeks when stored at 4°C. Incubation at 55°C for 12–16 hours resulted in nonviability of the organism (4). *R. anatipestifer* has been reported to survive in tap water and turkey litter for 13 and 27 days, respectively (6). It is sensitive to penicillin, novobiocin, chloramphenicol, lincomycin, enrofloxacin, ceftiofur, streptomycin, erythromycin, ampicillin, bacitracin, neomycin, and tetracycline but is resistant to kanamycin and polymyxin B (4,13). *R. anatipestifer* is relatively resistant to gentamicin.

Strain Classification

R. anatipestifer isolates have been serotyped using agglutination and agar-gel precipitin (AGP) reactions. Both of these tests involve surface antigens that are presumed to be polysaccharides (9). Plate agglutination is rapid and convenient; tube agglutination is favored over AGP as it is quantitative in terms of antibody titers.

To date, 21 serotypes have been reported. Based on agglutination reactions, Harry identified 16 serotypes (A through P), 4 of which (E, F, J, and K) were lost during storage, and he found serotypes G and N to be identical to serotypes I and O, respectively (7, 21). Seven serotypes (1 through 7) were differentiated using AGP reaction (9). Subsequently, Bisgaard (7) reported serotypes 1, 2, 3, 4, 5, and 6 to be serologically identical to Harry's types A, I/G, L, H, M, and B, respectively. He also suggested numerical designation of serotypes to avoid confusion and to standardize serotype nomenclature for recognition of new serotypes. He identified 2 new serotypes (12 and 13). Serotype 7 was reported to be identical to serotype O/N, and a new serotype (8) was isolated (65). Sandhu and Leister (66) revised the typing scheme proposed by Bisgaard. They redesignated Harry's serotypes C and D as types 9 and 10, excluded serotype 4, which was not RA, and reported 5 new serotypes (11, 14, 15, 16, 17). Loh *et al.* (42) reported serotypes 13 and 17 to be identical. They redesignated Harry's type P as serotype 4 and added 3 new serotypes (17, 18, and 19), which were isolated from ducks in Singapore. Two new serotypes (20, 21) were isolated from ducks in Thailand (51); one of these (serotype 20) was later excluded as it was not RA (59). A new serotype isolated from ducks in Thailand replaced serotype 20 (49). All serotypes reacted specifically with homologous-type antisera with the exception of serotype 5, which gave minor cross-reactions with serotypes 2 and 9 (42, 65).

Cell lysates of various serotypes showed many bands when subjected to polyacrylamide gel electrophoresis (29). Most of the bands were common to all serotypes, but some were specific to

individual serotypes. Subramaniam and his associates (71) cloned an outer membrane protein gene (*OmpA*) from RA that encoded for a 42-kDa major antigenic outer membrane protein (OmpA). All reference strains of RA had the *OmpA* gene, although some minor genetic differences were observed in different strains. Tsai *et al.* (72) reported that all of the RA strains fall into a single cluster based on the phylogenetic analysis of 16S rRNA gene and that the 16S rRNA gene-based polymerase PCR may be a suitable test for screening RA infections.

Recently, most of the RA strains were shown to contain plasmids (12). A 3.9b plasmid carried protein genes similar to the virulence-associated genes of other bacteria. An insertion sequence element found on a second plasmid may be of importance in epidemiological studies (75).

Pathobiology and Epidemiology

Incidence and Distribution

The disease occurs worldwide and has been recognized in countries that have intensive duck production (61). There is a wide variation in the severity of the disease depending on the strain of the organism, the age of the host, and the route of exposure (26, 67). Often, more than one serotype is responsible for the disease at a single farm or in the same hatch of birds.

Natural and Experimental Hosts

R. anatipestifer infection is primarily a disease of domestic ducks and geese. Naturally occurring outbreaks have been reported in turkeys (27, 78). Serious outbreaks in turkeys in the United States and other countries showed that RA is a potential pathogen of domestic turkeys (19, 47, 48, 70). *R. anatipestifer* has also been isolated from pheasants (11), chickens (57), guinea fowl and quail (48), partridge (77), and other waterfowl (17, 37, 46, 54, 76). Recently, it has also been isolated from gulls, budgerigars, guillemots, and pigs (32).

Chickens, geese, pigeons, rabbits, and mice were reported to be refractory to infection with RA; guinea pigs succumbed to inoculation of large doses intraperitoneally (20, 28). However, Heddleston (26) observed that 8×10^6 organisms inoculated into the foot pad killed 5 of 7 one-day-old chicks; 4×10^6 organisms in 2-week-old white Chinese goslings produced signs and lesions similar to those seen in Pekin ducklings.

Ducklings, 1–8 weeks of age are highly susceptible. Ducklings under 5 weeks of age usually die within 1–2 days after clinical signs appear; older birds may survive longer. The disease is rare in breeder ducks.

Transmission, Carriers, and Vectors

Infection takes place via the respiratory tract (38) or through wounds of the skin, particularly of the feet (2). RA and RA-like bacteria have been isolated from pharyngeal mucosa of clinically normal ducklings (58). Cooper (14) suggested that the disease in turkeys may be transmitted by arthropod vectors based on its seasonal occurrence and the apparent affinity of RA for host erythrocytes. The disease can be reproduced most consistently by injection of the organism intravenously, subcutaneously, intraperitoneally, intramuscularly, into the foot pad, or into the infraorbital sinus. Experimental infection by subcutaneous and intravenous routes caused high mortality; while no or low mortality was observed in ducklings infected by the oral or nasal route (3, 24, 67).

Incubation Period

The incubation period is usually 2–5 days. Artificial infection of ducklings by the subcutaneous, intravenous or infraorbital sinus routes results in clinical signs and deaths as early as 24 hours postinfection.

Clinical Signs

Signs most often observed are listlessness, ocular and nasal discharge, mild coughing and sneezing, greenish diarrhea, ataxia, tremor of head and neck, and coma. Affected ducklings lie on backs paddling their legs and are unable to move with the brood. Surviving ducks may be stunted (52). Adverse environmental conditions or concomitant disease often predispose birds to outbreaks of RA infection. Mortality may vary from 5 to 75%; morbidity is usually higher.

Pathology

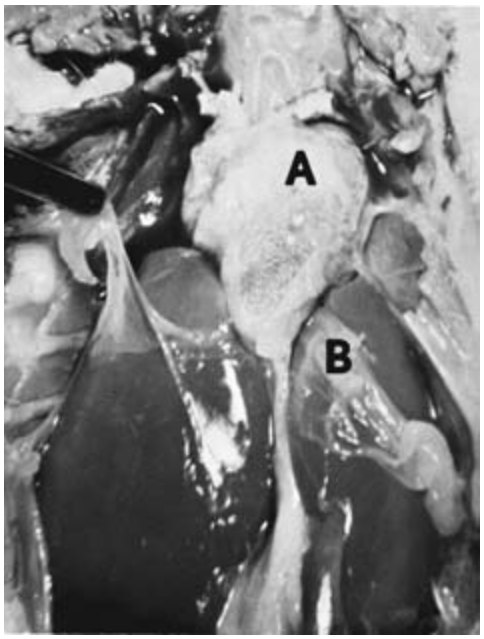
Gross

The most obvious gross lesion in ducks is fibrinous exudate, which involves serosal surfaces in general, but is most evident in the pericardial cavity, over the surface of the liver (Fig. 19.14), and in air sacs; similar lesions have been reported in turkeys and other birds. Fibrinous airsacculitis is common; both abdominal and thoracic air sacs may be involved. Spleen may be enlarged and mottled. Mucopurulent exudate in nasal sinuses and caseous exudate in oviducts have been observed (16).

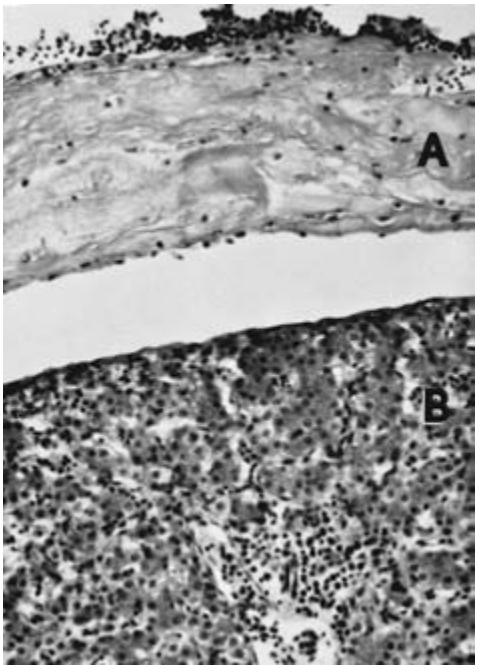
Chronic localized infections may occur under the skin and occasionally in joints. Skin lesions are in the form of necrotic dermatitis on the lower back or around the vent. Yellowish exudate is observed between skin and fat layers.

Microscopic

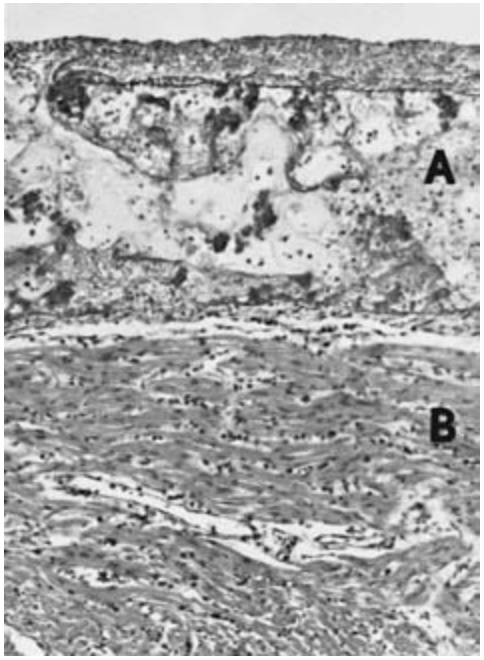
Fibrinous exudate on the heart contains a few inflammatory cells, primarily mononuclear cells, and heterophils (Fig. 19.15). Liver lesions observed in the acute stage of the disease are mild periportal mononuclear leukocytic infiltration, cloudy swelling, and hydropic degeneration of parenchymal cells (Fig. 19.16). In less acute cases, moderate periportal lymphocytic infiltration may be observed (52). In air sacs, mononuclear cells are the predominant cell type in the exudate. Multinuclear giant cells and fibroblasts may be observed in chronic cases (16). The respiratory tract may also be infected without showing clinical signs. Lungs of infected ducks may be unaffected; there is interstitial cellular infiltration and proliferation of lymphoid nodules adjacent to parabronchi (52); or there may be an acute fibrinopurulent pneumonia (20). Infections of the central nervous system can produce fibrinous meningitis. Jortner and his associates (36) studied lesions in the central nervous system of naturally infected ducklings and described diffuse fibrinous meningitis with leukocytic infiltration in and around the walls of meningeal blood vessels. Extensive exudate was observed in the ventricular system. Slight to moder-



19.14. *Riemerella anatipestifer* infection. Fibrinous epicarditis (A), pericarditis, and perihepatitis (B). Forceps hold exudate from surface of liver.



19.16. *Riemerella anatipestifer* infection. Fibrinous exudate (A) over surface of liver (B). H & E, ×300.



19.15. *Riemerella anatipestifer* infection. Fibrinous exudate (A) over surface of heart (B). H & E, ×150.

ate leukocytic and microglial infiltrates were observed in subpial and periventricular brain tissue. Lymphoid necrosis and depletion of lymphocytes have been observed in spleen and bursa of Fabricius (67).

Immunity

Ducklings that recover from the disease are resistant to subsequent infection (2, 20, 28). Inactivated bacterins have been used in ducks to prevent RA infection. Ducks vaccinated with formalin-inactivated bacterins and subsequently challenged with strains representing serotypes 1, 2, and 5 developed homologous, but not heterologous, protection. A trivalent bacterin containing these strains provided protection against challenge with each serotype, but the protection lasted only a short time (60). Harry and Deb (23) evaluated the effectiveness of several types of bacterins and conducted a field trial with a formalin-inactivated bacterin. A single dose of oil-emulsion bacterin provided longer lasting immunity in ducklings (18, 60). Cell-free culture filtrate has also been reported to provide significant protection against homologous challenge (50). Outer membrane proteins OmpA and P45 failed to provide protection against a virulent challenge but did result in production of RA-specific antibodies (34). One-day-old ducklings exposed to live avirulent strains by aerosol or through the drinking water were resistant when challenged at 3–6 weeks of age with virulent homologous strains (62). Passive protection of progeny may be obtained by immunization of the female breeder ducks; maternal immunity lasts for about 2–3 weeks (63). RA specific antibodies were detected in the egg yolk and sera of vaccinated breeder ducks (41); maternal antibodies in the progeny lasted up to 10 days of age. Cell-mediated immunity to RA antigens was transient (similar to vaccination with the bacterin), and live vaccine induced longer lasting protection (29, 62).

Diagnosis

Isolation and Identification of Causative Agent

Although a presumptive diagnosis may be made from clinical signs and necropsy findings, a definite diagnosis should be based on isolation and identification of RA. The bacterium can be isolated most readily when birds are in the acute stage of the disease. Suitable tissues for culture are brain, heart blood, air sacs, bone marrow, lung, liver, and exudates from the lesions. Samples should be taken aseptically, streaked on blood agar or trypticase soy agar containing 0.05% yeast extract, and incubated in a candle jar at 37°C for 24–72 hours. Addition of newborn calf serum (5%) and gentamicin (5 mg/1000 ml) to plate media is helpful for the isolation of RA from contaminated specimens. Isolated colonies should be selected for inoculation of the differential media and identified on the basis of characteristics described under "Etiology." Serotype identification can be established by agglutination and/or AGP reactions with specific antisera. Molecular fingerprinting by restriction endonuclease analysis and repetitive sequence PCR are useful to differentiate RA strains and may be helpful in epidemiological studies (35, 56).

Serology

Immunofluorescent procedures can be used to identify RA in tissue or exudate from infected birds (44). Agglutination test and enzyme-linked immunosorbent assay (ELISA) can be used to detect serum antibodies. ELISA is more sensitive than agglutination test but is not serotype-specific (25, 33, 41).

Differential Diagnosis

R. anatipestifer infection should be differentiated from other septicemic diseases caused by *Pasteurella multocida*, *Coenonia anatina*, *Escherichia coli*, *Streptococcus faecium*, and salmonellae. Because these diseases produce gross lesions indistinguishable from those caused by RA, diagnosis must include isolation and identification of the causal organism. Differential diagnosis should also include chlamydiosis, especially in turkeys and in areas where the latter is a serious problem.

Intervention Strategies

Management Procedures

The most important aspects of prevention are good biosecurity, management, and sanitation practices. This includes proper ventilation, especially in houses where ducks are raised in total confinement. Predisposing factors such as stress due to overcrowding or exposure to hot or cold weather should be avoided. Strict measures should be taken to prevent the spread of infection from diseased to healthy flocks. If ducks are raised on wire, the floors should be periodically washed and sanitized to avoid accumulation of manure and to reduce the exposure to infection.

Vaccination

Inactivated bacterins have been reported to prevent or reduce mortality due to RA (23, 38, 60). Because immunity induced by bacterins is serotype-specific, an ideal bacterin should contain cells of the predominant serotypes to provide an effective protection. A bacterin containing serotypes 1, 2, and 5 has been used in the United States and Canada. Ducklings are vaccinated at 2 and 3 weeks of age to provide adequate protection up to market age (38). A single inoculation of oil-emulsified bacterin has been reported to produce longer-lasting protection, but it may cause unfavorable lesions at the site of inoculation (18, 60).

A live RA vaccine, developed against serotypes 1, 2, and 5, provided significant protection against experimental or field infections with virulent organisms when administered to 1-day-old ducklings by aerosol or in drinking water (62). A single vaccination protected ducklings up to at least 42 days of age. The vaccine strains grew in the upper respiratory tract and produced a humoral antibody response. The vaccine was demonstrated to be avirulent to 1-day-old ducklings when administered by aerosol or injection into the infraorbital sinus. The vaccine strains were safe in ducks up to 10 back-passages using the contact-exposure method. Breeder ducks can be vaccinated with the bacterin or live vaccine to provide protection in progeny through maternal immunity that may last up to 2–3 weeks of age. Maternally immune ducklings respond successfully to active immunization with a live or inactivated vaccine (63).

Treatment

Antibiotics and sulfa drugs have been tested for treatment of RA with varying degrees of success. Sulfamethazine, 0.2–0.25%, in drinking water or feed, was reported to prevent the onset of clinical signs in ducks exposed experimentally to RA (2). Sulfaquinoxaline at levels of 0.025 or 0.05% in feed was effective in reducing mortality in field and experimental infections (15, 64). Medicated feeds containing novobiocin (0.0303–0.0368%) or lincomycin (0.011–0.022%) were reported to be highly effective in reducing mortality when started 3 days prior to experimental infection. A combination of sulfadimethoxine and ormetoprim, when administered at 0.02–0.12% levels in feed, prevented or reduced mortality and gross lesions in experimentally exposed ducks (45, 64). Tetracyclines were of little value for treatment of RA infection (1, 64). Subcutaneous injection of lincomycin-spectinomycin, penicillin, or a combination of penicillin and dihydrostreptomycin were reported to be effective in reducing mortality in artificially infected ducklings (64). Enrofloxacin has been shown to be highly effective in preventing mortality in ducklings when given in drinking water at levels of 50 ppm for the first day followed by 25 ppm for the next 4 days (73). Ceftiofur, a broad-spectrum cephalosporin, reduced mortality in experimentally infected ducklings given a single dose of 2 mg/kg bodyweight subcutaneously 5 hours after infection (13).

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Ornithobacterium rhinotracheale Infection

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Introduction

Definition and Synonyms

Ornithobacterium rhinotracheale infection is a contagious disease of birds that causes respiratory distress, mortality, and decreased growth. The severity of clinical signs, duration of the disease, and mortality are extremely variable and are influenced by environmental factors such as poor management, inadequate ventilation, high stocking density, poor litter conditions, and poor hygiene.

Economic Significance

O. rhinotracheale can be associated with high economic losses in poultry due to increased mortality and condemnation rates, decreased egg production, or decreased growth.

Public Health Significance

Currently, *O. rhinotracheale* has not been found to be of any public health significance.

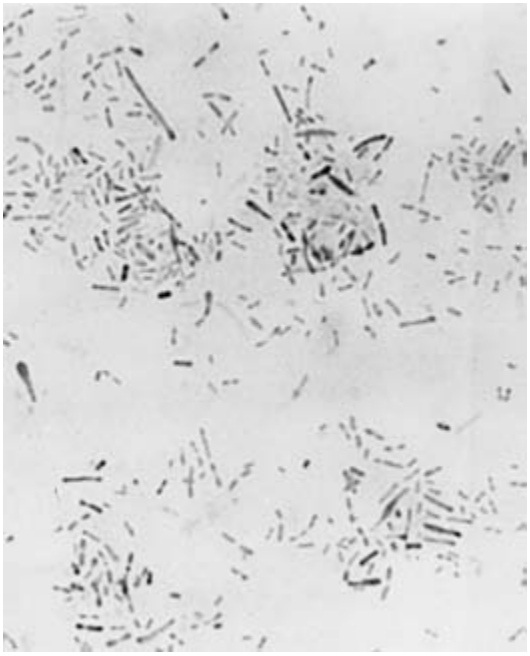
History

O. rhinotracheale was first characterized in 1993 by Charlton *et al.* (17). The following year, Vandamme *et al.* (104) reported the phylogenetic position and various genotypic, chemotaxonomic, and classical phenotypic characteristics of 21 isolates, and proposed the name *O. rhinotracheale*. However, it appears that this bacterium was isolated and studied prior to 1993 (44).

In 1981, the first known isolation of *O. rhinotracheale* was made in northern Germany from 5-week-old turkeys with nasal discharge, facial edema and fibrinopurulent airsacculitis. In 1983, it was cultured from the tracheas of young rooks. In 1986, *O. rhinotracheale* was isolated from turkeys in Israel of various ages with acute exudative pneumonia and airsacculitis (12). In 1987, *O. rhinotracheale* was isolated from 10-week-old Pekin ducks in Hungary with a fowl cholera-like disease (89). Between 1986 and 1988, *O. rhinotracheale*, at that time identified as *Pasteurella*-like bacterium, was isolated from turkey breeding flocks in England which showed general depression, decreased egg production, coughing, low mortality, and fibrinous airsacculitis and pneumonia (107).

Isolations of *O. rhinotracheale* in California began in 1986, and Charlton characterized 14 isolates collected from 1990 to 1991 from turkeys and chickens with respiratory disease (17). In 1991, Du Preez observed a respiratory disease in broilers in South Africa from which *O. rhinotracheale* was isolated (87). *O. rhinotracheale* was reported to cause fowl cholera-like lesions in turkeys in Germany in 1993 and 1994 (37, 43) and 32-week-old breeder turkeys in the United States in 1996 (20).

Since its identification by Vandamme, *et al.*, in 1994, *O. rhinotracheale* has been isolated from birds in various countries throughout the world.



19.17. *Ornithobacterium rhinotracheale* showing highly pleomorphic nature. Gram stain of bacteria from a 48-hour culture. Gram stain, ×375. (Charlton)

Etiology

Classification

Name and Synonyms

O. rhinotracheale belongs to the rRNA superfamily V within the Cythophaga-Flavobacterium-Bacteroides phylum, and is closely related to two other poultry bacteria, *Riemerella anatipestifer* and *Coenonia anatina* (104, 105). Previously, the bacterium was designated as *Pasteurella*-like, *Kingella*-like, Taxon 28, or pleomorphic gram-negative rod before the name *Ornithobacterium rhinotracheale* gen. nov. sp. nov. was suggested (9, 57, 104).

Morphology and Staining

O. rhinotracheale is a gram-negative, nonmotile, highly pleomorphic, rod-shaped, nonsporulating bacterium. From agars, it appears as short, plump rods measuring 0.2–0.9 μm in width and 0.6–5 μm in length (Fig. 19.17). But from fluid media, very long rods measuring up to 15 μm can be observed.

Growth Requirements

O. rhinotracheale grows aerobically, microaerobically, and anaerobically. The optimal growth temperature is 37°C; however growth can occur at 30–42°C. The bacteria will grow best on 5–10% sheep blood agar, but readily grows on tryptose soy agar and chocolate agar. No growth occurs on MacConkey agar, Endo

agar, Gassner agar, Drigalski agar, or Simmons citrate media. The growth in fluid media can be strain-dependent, and media such as brain heart infusion broth, Pasteurella broth or, Todd Hewitt broth are needed.

Colony Morphology

O. rhinotracheale develop very small, nonhemolytic colonies that are circular, gray to gray-white, sometimes with a reddish glow, and convex with an entire edge. On primary isolation, the colonies of most *O. rhinotracheale* cultures show great differences in size (1–3 mm after 48 hour incubation) but when subcultured, the colony size will become more uniform.

Biochemical Properties

Conventional biochemical tests can be inconsistent. Phenotypic characteristics include the production of oxidase, lack of catalase production, lack of motility, no reaction on triple sugar iron agar, production of beta-galactosidase, the inability to reduce nitrate to nitrite, and the inability to grow on MacConkey agar. There is one report of a cytochrome oxidase-negative strain of *O. rhinotracheale* isolated from turkeys in Germany (67). Predominant fatty acids detected are 15:0 iso, 16:0, 15:0 iso 3OH, 17:0 iso, 16:0 3OH, 17:0 iso 3OH, and unknown peaks with equivalent chain lengths of 13.566 and 16.580. Enzymatic reactions are listed in Table 19.4.

Table 19.4. Enzyme production of *O. rhinotracheale.*

Test	Result
Alkaline phosphatase	+
Esterase lipase	+
Leucine aminopeptidase	+
Valine aminopeptidase	+
Cystine aminopeptidase	+
Acid phosphatase	+
Phosphohydrolase	+
α-Galactosidase	+
ß-Galactosidase	+
α-Glucosidase	+
N- Acetyl-beta-glucosamidase	+
Trypsin	+
α-Chymotrypsin	+
Lipase	–
ß-Glucuronidase	–
ß-Glucosidase	–
α-Mannosidase	–
α-Fucosidase	–

Susceptibility to Chemical and Physical Agents

O. rhinotracheale strains were completely inactivated by a 0.5% solution containing formic and glyoxyl acid, and a 0.5% solution of an aldehyde-based (20% glutaraldehyde) product after 15 minutes exposure time (40). These preparations were able to inactivate *O. rhinotracheale in vitro* at concentrations of 0.5% within 15 minutes.

Antigenic Structure and Toxins

Currently, no special structures or properties such as pili, fimbriae, plasmids (49), or specific toxic activities have been reported.

Strain Classification

Antigenicity

Using boiled extract antigens (BEAs) and monovalent antisera in the agar gel precipitation (AGP) and enzyme-linked immunosorbent assay (ELISA) tests, 18 serotypes (A through R) of *O. rhinotracheale* have been determined (92, 95). Serotype A was the most prevalent serotype among chicken isolates (97%) and turkey isolates (61%) (95). There appears to be a correlation between the geographic origin of the *O. rhinotracheale* isolates and their serotype. Serotype C could be isolated only from chickens and turkeys in South Africa and United States (33, 90, 95). There is no indication of host specificity of the serotypes.

Hafez and Sting (41) compared the efficacy of using different antigen extractions (heat-stable, proteinase K-stable and sodium dodecyl sulfate) for serotyping *O. rhinotracheale* in the AGP and ELISA tests. Results indicate that the AGP test with heat-stable or proteinase K-stable antigen extractions is a suitable method for serotyping. Numerous cross-reactions were seen with the ELISA making it unreliable for serotyping.

Immunogenicity or Protective Characteristics

Using a novel experimental method of combining immune depletion and passive transfer of immunity within the same host, Schuijffel, *et al.*, found that the antibody-mediated immunity in chickens was a key component in the protection against *O. rhinotracheale* infection (72).

Molecular

Amonsin *et al.* (3) using multilocus enzyme electrophoresis, repetitive sequence based-PCR, and 16S rRNA gene sequencing demonstrated that the majority of 55 *O. rhinotracheale* isolates recovered from domesticated poultry throughout the world had limited heterogeneity and were represented by a small group of closely related clones. They propose that the bacterium was recently introduced to domesticated poultry from wild bird populations.

Twenty-three isolates of *O. rhinotracheale* from France were tested using the random amplified polymorphic DNA (RAPD) analysis (49). Results showed that this method gave reproducible DNA fingerprints and a good level of discrimination, thus appearing to be another method for typing.

Van Empel *et al.* (89) used amplified fragment length polymorphism (AFLP) to characterize 56 isolates belonging to different serotypes which were isolated from different bird species from various countries. These isolates could be grouped in 3 major clusters with significantly different DNA fingerprints.

Popp and Hafez (62) investigated several *O. rhinotracheale* isolates from turkeys and chickens originated from Germany,

Hungary, and Spain by pulsed-field gel electrophoresis (PFGE) of genomic macro-restriction fragments using the enzyme *Sal*I. In general, most isolates showed differences in DNA fingerprints although the overall profiles were very similar and a correlation between geographic origin, serotype and DNA fingerprint pattern was observed. In contrast, Koga and Zavaleta (48) recently investigated 25 *O. rhinotracheale* isolates from broilers, breeders, and layers from several geographic zones of Peru using PCR and repetitive extragenic palindromic PCR (rep-PCR) techniques. All 25 isolates tested had a genetic profile similar to that of the *O. rhinotracheale* type strain (American Type Culture Collection 51463) that was isolated from a turkey in the United Kingdom.

Pathogenicity

Pathogenicity differences appear to exist between isolates of *O. rhinotracheale*. Three South African *O. rhinotracheale* field isolates inoculated into the caudal abdominal air sacs of 28-day-old broiler chickens showed significant differences in the production of airsacculitis and arthritis (85). In addition, van Veen *et al.* (102) found that Dutch and South African isolates were more pathogenic than an American isolate in broiler chickens when aerosol challenged.

The pathogenicity of 119 isolates from turkeys and chickens using the embryo lethality test were studied (39). This study showed that the inoculation of about 500 cfu of *O. rhinotracheale* into the allantoic sac of 11-day-old embryos discriminated between pathogenic and nonpathogenic *O. rhinotracheale* isolates. On the basis of death rate, isolates considered to be nonpathogenic had an embryo death rate 10–20%, moderately pathogenic had a 21–60% embryo death rate, and highly pathogenic isolates had a death rate of >60%.

Soriano *et al.* found *in vitro* adherence of *O. rhinotracheale* isolates to chicken tracheal epithelial cells (76).

Pathobiology and Epidemiology

Incidence and distribution

After being recognized in 1994, numerous isolations of *O. rhinotracheale* have been reported throughout the world (1, 2, 4, 11, 13, 14, 16, 21, 24–26, 31, 32, 35, 37, 45, 47, 49, 58, 60, 66, 70, 75, 76, 80, 83, 84, 108).

Natural and Experimental Hosts

O. rhinotracheale has been isolated throughout the world from numerous bird species, including chicken, chukar partridge, duck, goose, guinea fowl, gull, ostrich, partridge, pheasant, pigeon, quail, rook and turkey (17, 97, 104). In commercial poultry, all ages appear to be susceptible, although pathogenicity appears more significant in older birds.

Many case reports of *O. rhinotracheale* infection report a concomitant infection with other respiratory pathogens, such as *Escherichia coli* (19, 70), *Bordetella avium* (16), Newcastle disease virus (84), infectious bronchitis virus (26, 60, 71), avian metapneumovirus (8, 33, 46, 55), *Mycoplasma synoviae* (108) and *Chlamydophila psittaci* (98). Most experimental studies have concluded that, when experimentally inoculated by itself, *O. rhinotracheale* causes minimal pathologic lesions in chickens and turkeys and that the severity of lesions are enhanced when there is a concurrent infection with respiratory viruses or bacteria (5, 19, 22, 28, 94, 96, 102).

However, some studies report production of pathologic lesions similar to those seen in field cases in chickens and turkeys using *O. rhinotracheale* alone (69, 79, 85, 102).

Transmission, Carriers, and Vectors

O. rhinotracheale appears to spread horizontally by direct and indirect contact through aerosols or drinking water. *O. rhinotracheale* was found to survive 1 day at 37°C, 6 days at 22°C, 40 days at 4°C, and at least 150 days at –12°C (53). The survival of *O. rhinotracheale* at lower temperatures may be associated with the higher incidence of reported infections during the winter months. It did not survive 24 hours at 42°C.

There is circumstantial evidence that vertical transmission occurs (89, 103). In addition, *O. rhinotracheale* has been isolated from the ovaries, oviduct, hatching eggs, infertile eggs, dead embryos, and dead-in-shell chickens and turkeys (5, 23, 59, 83, 89). However, when *O. rhinotracheale* was inoculated into embryonated chicken eggs, the embryos were killed by the ninth day and *O. rhinotracheale* was not isolated from the eggs suggesting it is not transmitted via eggs during hatching (106).

Incubation Period

Experimental inoculation of 22-week-old turkeys with *O. rhinotracheale* resulted in depression, coughing and decreased feed intake within 24 hour (79). In 48 hour, turkeys were coughing bloody mucus. Five days PI, the coughing had decreased and the surviving turkeys were less depressed.

In experimental infections in 5-week-old chickens, *O. rhinotracheale* infected the respiratory organs within 2 days postinoculation and clinical signs were seen after 4 days (96).

Clinical Signs

The severity of clinical signs, duration of the disease and mortality of *O. rhinotracheale* outbreaks are extremely variable. They can be influenced by many environmental factors such as poor management, inadequate ventilation, high stocking density, poor litter conditions, poor hygiene, high ammonia levels, concurrent diseases and the type of secondary infection.

Clinical signs in broiler chickens generally appear at 3–6 weeks of age with a mortality rate of 2–10% showing depression, decrease in food intake, reduced weight gains, and transient nasal discharge and sneezing, followed by facial edema (28, 60, 97). *O. rhinotracheale* can also cause sudden death (up to 20% in a couple of days) in young birds with infections of the brains and skull (29) with or without respiratory symptoms.

In broiler breeders the disease affects the birds in the laying period, primarily at the peak of production or soon before entering production. There is a slight increase in mortality, a decrease in feed intake, and some mild respiratory symptoms. Mortality is variable and relatively low in uncomplicated cases. There can be a drop in egg production, decrease in egg size, and poor eggshell quality. Fertility and hatchability are unaffected in many cases (31).

In commercial laying-type chickens, decreased egg production, increased misshapen eggs, and increased mortality have been associated with *O. rhinotracheale* infection (80).

Roepke (66) found a higher severity of clinical signs and mortality in older turkeys, and the majority of young infected flocks appeared clinically normal. In many cases young poults are affected between 2 and 8 week of age (16). Normal mortality ranges between 1–15% during the acute phase (8 days), but infections can be accompanied with mortality rates of up to 50% (6, 20). Initial symptoms are coughing, sneezing, and nasal discharge followed, in some cases, by severe respiratory distress, dyspnea, prostration, and sinusitis. These symptoms are accompanied with a reduction in feed consumption and water intake. In turkey breeder flocks, there can also be a decrease in egg production and an increase in the number of unsuitable hatching eggs (20, 97).

O. rhinotracheale has been reported to cause neurological signs or paralysis through arthritis, meningitis, osteitis, and osteomyelitis in chickens and turkeys (25, 82, 97).

Pathology

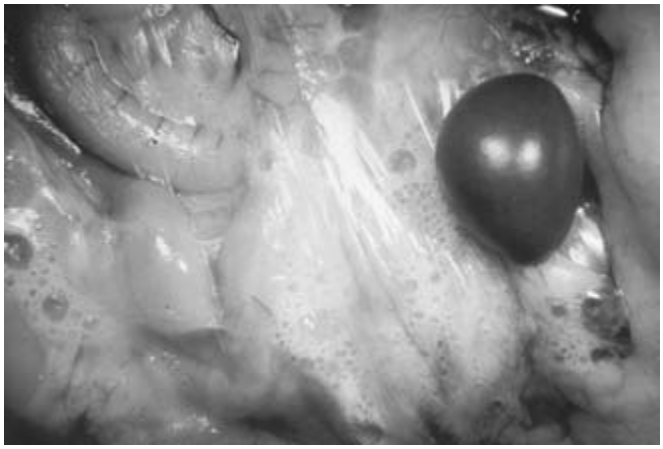
Gross

In broiler chickens, the common gross lesions include pneumonia, pleuritis, and airsacculitis. At slaughter or post-mortem examination, foamy, white, yogurt-like exudate can be seen in the air sacs (predominantly abdominal) (Fig. 19.18), most of the time accompanied by unilateral pneumonia (94). Lesions caused by *O. rhinotracheale* can lead to condemnation rates of 50% or more (99). In addition, subcutaneous edema over the cranium with adjacent osteitis, osteomyelitis and encephalitis has been reported in chickens (29, 30, 78).

In turkeys, there is edema and unilateral or bilateral consolidation of the lungs with fibrinous exudate on the pleura (Fig. 19.19). In addition, there could be fibrinosuppurative airsacculitis, pericarditis, peritonitis, and mild tracheitis. In some cases, swelling of the liver and spleen, as well as degeneration of heart muscles, has been observed (37). Infections of the joints and vertebrae can be seen in older birds.

Microscopic

Most histologic lesions can be seen in the lungs, pleura, and air sacs. In field cases, the lungs (Fig. 19.20) are congested, and throughout the parenchyma, there are large collections of fibrin admixed with macrophages and heterophils lying free within the lumen of air capillaries and parabronchi. There are pronounced and diffuse interstitial infiltrates of macrophages with smaller numbers of heterophils. There are widespread coalescing foci of necrosis often centered within the lumen of parabronchi with extension of the necrosis into the adjacent parenchyma. These necrotic foci usually are filled with dense aggregates of necrotic heterophilic infiltrate or exudate, and there can be scattered small clusters of bacteria seen within the necrotic foci. Numerous blood capillaries can be distended and filled with fibrin thrombi. The pleura and air sacs can be severely thickened and edematous with interstitial fibrin deposits, diffuse heterophilic infiltrate, scattered small foci of necrotic heterophilic infiltrate, and fibrosis.



19.18. Thickened, opaque air sacs with profuse, foamy, white to yellow, "yogurt-like" exudate associated with infection of *Ornithobacterium rhinotracheale* in 36-day-old broiler chickens. Possible concurrent infection with infectious bronchitis virus. (Salem)



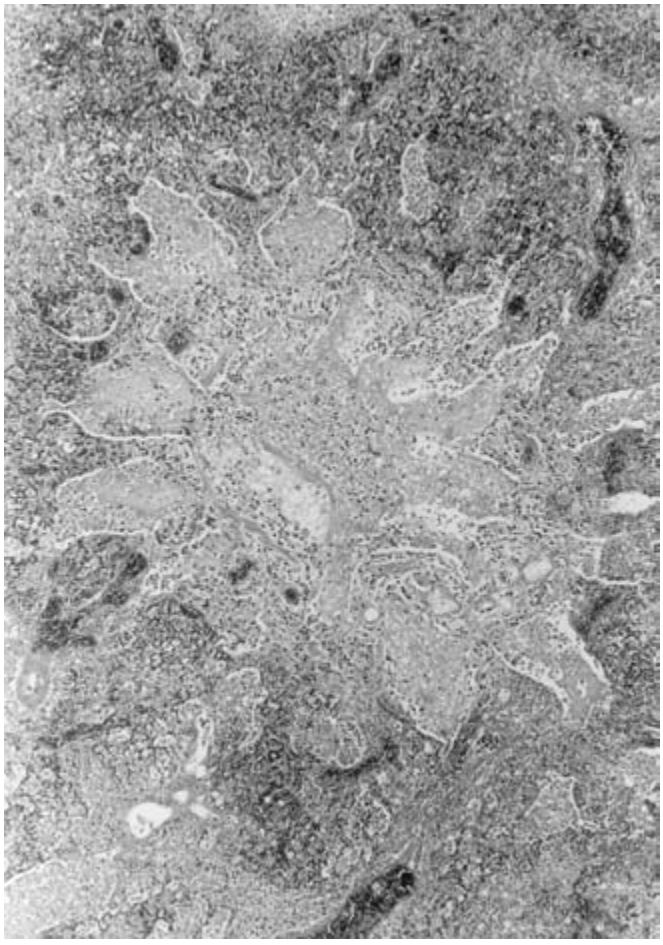
19.19. Pneumonia and pleuritis associated with *Ornithobacterium rhinotracheale* infection in 31-week-old turkey. (Shivaprasad)

Immunity

Minimal information is available regarding immunity to *O. rhinotracheale*. The active immunity induced by inactivated vaccines was found to be serotype specific, but live vaccination can induce a degree of cross-protection between some serotypes (74). Passive immunity can be induced for up to 3–4 weeks by maternally derived antibodies.

Diagnosis

It is difficult to make a presumptive diagnosis based on clinical signs and necropsy findings. A definitive diagnosis must be



19.20. Severe fibrinoheterophilic inflammation of lung associated with *Ornithobacterium rhinotracheale* infection in 31-week-old turkey. H & E. ×15. (De Rosa)

based on the isolation and identification of *O. rhinotracheale* and/or detection of antibodies.

Isolation and Identification of Causative Agent

Bacterial Isolation and Identification

The trachea, lungs, and air sacs are the best tissues from which to isolate *O. rhinotracheale*. The infraorbital sinus and nasal cavity are also suitable sites for culture, but *O. rhinotracheale* can be masked easily by the overgrowth of other bacteria. Culturing heart blood and liver tissue under field condition has revealed negative results (37), although it has been isolated from those organs, as well as joints, brain, ovary and oviduct after experimental infections (5, 87).

O. rhinotracheale can be isolated on common, nonselective blood or chocolate agar. Colonies grow in 24 hours, but it is best to hold inoculated plates for 48–72 hours in air enriched with 7.5–10% CO_2. Colonies will appear pinpoint to small (approximately 1–2 mm diameter), gray to gray-white, circular, and convex with an entire edge. Gram stain will reveal characteristic pleomorphic gram-negative bacteria. Colonies are catalase-negative and oxidase-positive. Pure *O. rhinotracheale* cultures have a distinct odor, similar to that of butyric acid. Additional testing is necessary to confirm the identification of *O. rhinotracheale.*

In contaminated samples with fast growing bacteria, such as *E. coli*, *Proteus* sp. or *Pseudomonas* sp., *O. rhinotracheale* colonies may be overgrown and are difficult to detect in routine investigation. Because it has been shown that most *O. rhinotracheale* isolates are resistant to gentamicin (5), Back recommended the use of 10 µg of gentamicin per ml of blood agar medium in an effort to isolate *O. rhinotracheale* from contaminated samples. Blood agar containing 5 µg per ml of gentamicin and polymyxin B was also effective (88).

The API-20NE system (bioMérieux, France) was found useful for the identification of *O. rhinotracheale* (95). Ninety-nine percent of isolates were found to have biocodes 0-2-2-0-0-0-4 (65%) or 0-0-2-0-0-0-4 (34%). For those isolates that were positive for arginine dihydrolase test, biocodes 0-3-2-0-0-0-4 or 0-1-2-0-0-0-4 were found.

The API-ZYM system (bioMérieux, France) can be used to determine the enzymatic activity. In this system, the consistent findings are negative reactions for lipase, ß-glucuronidase, ß-glucosidase, α-mannosidase and α-fucosidase (18).

Another rapid commercial gram-negative bacterial identification system was used to investigate 110 *O. rhinotracheale* strains and found to be a suitable adjunct to classical tests (63).

The rapid slide agglutination test has been used for diagnostic purposes (11, 12). In a study of 112 isolates, however, autoagglutinable strains were regularly found (92).

The agar-gel precipitation test (AGP), using known positive antisera, can be used to identify and serotype *O. rhinotracheale* isolates (95).

Another method used for the identification of suspect isolates is the PCR (34, 45, 89).

Antigen Detection

PCR was used for the detection of *O. rhinotracheale* in tracheal swabs of heavily infected broilers (45). In addition, immunofluorescence antibody test (92) and immunohistochemical staining were used to detect *O. rhinotracheale* in chickens (99). Subsequently, Van Veen *et al.* (101) found that the immunofluorescence assay and the peroxidase-antiperoxidase test were equally sensitive. Using these tests, they were able to identify a higher percentage of *O. rhinotracheale* infected chicken broiler flocks at slaughter when compared with conventional diagnostic methods, i.e., serology and/or bacteriology.

Serology

Serology is useful for flock monitoring or as an aid in the diagnosis of *O. rhinotracheale* infection.

The serum plate agglutination test (SPAT) has been used as a rapid test for the detection of antibodies against *O. rhinotracheale* (12, 27, 42). One SPAT was developed using a nonserotyped Minnesota isolate of *O. rhinotracheale* and reported to have good sensitivity and susceptibility (6). However, in another study (52) the SPAT detected only 65% of infected birds during

the first 2 week of infection and declined significantly thereafter. This suggests that the SPAT detects IgM antibodies, which are efficient in agglutination with specific antigens. In addition, most SPAT-reactions are serotype-specific, although cross-reactions do occur (91).

ELISAs have been developed using different serotypes and extracted antigens of *O. rhinotracheale*. Boiled extract antigens, which are used for serotyping, tend to give the best results for serotype-specific tests (95). Conversely, SDS-antigen extraction (38) and extracted outer membrane proteins of *O. rhinotracheale* (52) will result in more cross-reactions allowing detection of antibodies against different serotypes with one test. Field surveys using these ELISAs or commercial ELISA kits (available in Europe) have been useful for monitoring flocks and the diagnosis of *O. rhinotracheale* infections (7, 38, 42, 64, 68, 70, 86, 95).

Erganis *et al.* (27) developed a dot immunobinding assay (DIA) which appeared to be less sensitive than other agglutination tests.

Popp and Hafez (61) investigated the effect of amoxicillin treatment on the antibody kinetics following experimental infection. They found that immediate treatment did not influence the antibody response, whereas treatment that started 7 days PI resulted in a lower antibody response.

Differential Diagnosis

Respiratory lesions associated with *O. rhinotracheale* are similar to those caused by numerous bacteria, such as *E. coli, Pasteurella multocida*, *R. anatipestifer*, *Avibacterium paragallinarum* and *Chlamydophyla psittaci.*

Intervention Strategies

Management Procedures

O. rhinotracheale appears to be highly contagious and strict biosecurity measures should be followed to prevent its introduction into a flock. However, after a ranch is infected, *O. rhinotracheale* becomes endemic, especially in multiple-age farms and in areas with intensive poultry production (40, 66).

Vaccination

Vaccination of broiler chickens with injectable, inactivated vaccines was found effective (93, 103), but is probably impractical in most commercial flocks. Vaccination of broiler breeders with inactivated vaccines stimulated the development of high maternal antibodies (10, 15) which were sufficient to protect progeny against experimental challenge for up to 4 week of age (93) and produced lower mortality and condemnation rates in the progeny from vaccinated breeders (15). In addition, using a sprayed, live vaccine, at 14 days of age, resulted in the lowest percent of airsacculitis and pneumonia in challenged birds.

Schuijffel *et al.* (73) demonstrated that cross-protective immunity against different *O. rhinotracheale* serotypes can be induced by live vaccination in chickens. The genes encoding 8 cross-reactive antigens were amplified, cloned in an expression vector, and expressed in *E. coli*. Purified recombinant proteins with a molecular mass ranging from 35.9–62.9 kDa were mixed and tested as a subunit vaccine for protection against challenge with homologous and heterologous *O. rhinotracheale* serotypes. Subunit vaccination resulted in the production of antibodies reactive to the recombinant proteins on Western blot, and this eight-valent vaccine provided both homologous and heterologous protection against *O. rhinotracheale* challenge in chickens. In a subsequent study (74), they found that these 8 antigens are highly conserved among different *O. rhinotracheale* serotypes, but the different antigens were not expressed by all serotypes. In addition, their four component subunit vaccine was able to protect chickens against challenge with a heterologous *O. rhinotracheale* serotype.

Sprenger *et al.* (81) vaccinated 6-week-old turkeys either intranasally with a live vaccine or subcutaneously with a killed *O. rhinotracheale* vaccine, and challenged them intratracheally with live *O. rhinotracheale* at 14 or 21 week of age. Airsacculitis and pneumonia occurred less frequently in vaccinated birds than in unvaccinated birds after challenge, and *O. rhinotracheale* was recovered from unvaccinated, challenged birds but not from vaccinated, challenged or from unchallenged birds.

Field trails using monovalent or trivalent bacterins in meat turkey flocks resulted in production of antibodies for a short duration (36). In addition, the mortality and condemnation rates were lower in the vaccinated group compared to the unvaccinated group.

A temperature-sensitive mutant of *O. rhinotracheale* was developed (51) and used as a live vaccine in turkeys (50). Turkeys were vaccinated at 5 days of age via the drinking water, and challenged 7 weeks post-vaccination. Vaccinated birds had a significantly lower mean score for gross lesions when compared to unvaccinated birds, as well as a lower rate of reisolation and number of colony forming units of *O. rhinotracheale* per gram of lung tissue.

Vaccination of young turkeys with autogenous bacterins successfully reduced the number of outbreaks of *O. rhinotracheale* infections in turkeys in Israel (11).

Roepke (65) administered an autogenous live vaccine (oral route) in 6-week-old turkeys that resulted in a decrease in pathologic lesions and mortality when the birds were older. It is interesting to note that the birds were simultaneously spray-vaccinated with a live avian paramyxovirus 1 vaccine without any problems.

Due to the possibility of infection by several serotypes, it may be necessary to use different serotypes in the vaccines.

Treatment

The treatment of *O. rhinotracheale* infections with antibiotics is very difficult because of the variable susceptibility of strains. *O. rhinotracheale* can acquire reduced susceptibility or resistance against antibiotics such as amoxicillin, ampicillin, doxycycline, enrofloxacin, flumequine, gentamicin, lincomycin, trimethoprim-sulfonamide, tetracycline and tylosin (21, 54, 56, 77, 97, 100).

Susceptibility can be dependent on the regime used by the poultry industry in various geographical locations. For example, in countries were eggs are regularly dipped in an antibiotic such as enrofloxacin almost all isolates will show resistance to that antibiotic (89).

In 1996, Hafez reported that water medication using amoxicillin at a dose of 250 ppm for 3–7 days gave satisfactory results in most cases, and application of chlortetracycline at a dose of 500 ppm in drinking water for 4–5 days appeared to be effective (31). However, recent studies have shown that treatment with amoxicillin is no longer efficacious (56). In some cases, injections with various tetracyclines and penicillins were found to be effective.

Sixty-eight *O. rhinotracheale* isolates from the United States were found susceptible to ampicillin, erythromycin, penicillin, spectinomycin, and tylosin, and 54 of the 68 isolates were susceptible to neomycin, sarafloxacin, and tetracycline (59). It was also found that German isolates had a significantly lower percentage susceptible to erythromycin and sarafloxacin when compared with isolates from the United States.

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Bordetellosis (Turkey Coryza)

Mark W. Jackwood and Y. M. Saif

Introduction

Definition and Synonyms

Bordetellosis in poultry is a highly contagious upper respiratory tract disease caused by *Bordetella avium*. The disease is still commonly referred to as turkey coryza. Other synonyms that have been largely abandoned are alcaligenes rhinotracheitis (ART), adenovirus-associated respiratory disease, acute respiratory disease syndrome, *Bordetella avium* rhinotracheitis (BART), and turkey rhinotracheitis. The numerous names used for this disease reflect the initial confusion that has surrounded its etiology.

Economic Significance

Colonization of ciliated epithelium by *B. avium* results in protracted inflammation and distortion of the respiratory mucosa. In young turkeys, the disease is characterized by sneezing, mouth breathing, altered vocalization, stunted growth, clear oculonasal discharge, submandibular edema, tracheal collapse, and a predisposition to other infectious diseases. In chickens, the disease is similar, but *B. avium* appears to be an opportunistic pathogen. A careful analysis of the economic impact of bordetellosis has not been made; however, impaired growth and mortality resulting from secondary colisepticemia probably cause several million dollars in losses annually to the turkey industry in the United States.

Public Health Significance

Members of the *Bordetella* genus are well known for their capacity to colonize ciliated epithelium and produce respiratory disease in vertebrates. Despite similarities between whooping cough of humans (caused by *B. pertussis*) and bordetellosis of turkeys, there is no evidence that *B. avium* can either colonize or produce disease in humans (42).

History

Turkey rhinotracheitis (coryza) attributable to a bacterium of the genus *Bordetella* was first reported by Filion *et al.* (40) from Canada in 1967. Nearly a decade later, a similar syndrome was recognized, and in Germany the causative agent was identified as *Bordetella bronchiseptica*-like (56); whereas in the United States, it was associated with a respiratory adenovirus (106). In the United States, adenoviruses frequently were associated with the disease (21), but attempts to reproduce it experimentally often failed (32, 117). The postmortem finding of bursal atrophy led to speculation that infectious bursal disease virus (IBDV) may have a role in turkey rhinotracheitis (100, 118). Turkeys inoculated experimentally with IBDV failed, however, to develop clinical disease or lesions, and concurrent inoculation with IBDV and *B. avium* failed to exacerbate experimental bordetellosis (70). Other infectious agents, including mycoplasmas, paramyxoviruses, Yucaipa virus, and chlamydia (2), have been considered in the etiology of turkey rhinotracheitis (85). In 1980, the etiologic agent was reported to be *Alcaligenes faecalis* (110), and two types of the bacterium were observed and designated type I and type II. Then, in 1984, following phenotypic, serologic, and nucleic acid characterization, the name *Bordetella avium* was proposed and generally accepted (78). In addition, it was shown that *Alcaligenes faecalis* type I isolates were the same as *B. avium*, and *A. faecalis* type II isolates were designated *B. avium*-

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like to differentiate them from the etiologic agent of bordetellosis (68, 71). Further characterization of *B. avium*-like isolates led researchers to designate that organism as *Bordetella hinzii* (129). *Bordetella hinzii* has been associated with septicemia and bacteremia in older or immune-compromised people (30, 76) and in one patient with cystic fibrosis (41). Finally, in 1985, an acute, highly contagious upper respiratory disease of turkeys was recognized in England and Wales (3). The cause of that disease, which also has been called turkey rhinotracheitis, has been shown to be a pneumovirus (28) (see "Pneumovirus Infection," Chapter 3).

Etiology

Classification

Bordetellosis of turkeys is caused by *Bordetella avium* alone or in combination with environmental stresses and other respiratory pathogens. Experimental transmission (105) of the disease to susceptible poults by Simmons *et al.* (117) in the United States clearly established the etiologic agent as a small gram-negative bacillus. The bacterium, tentatively identified as *Alcaligenes faecalis*, closely resembled *Bordetella bronchiseptica*, except for its failure to split urea. A systematic study by Kersters *et al.* (78) compared 28 pathogenic turkey isolates from diverse sources with 50 culture-collection strains of closely related bacteria. Kersters *et al.* based their conclusions on morphologic, physiologic, nutritional, serologic, electrophoretic, and DNA-RNA hybridization and determined that the bacterial cause of turkey rhinotracheitis represented a new species of *Bordetella*; the name *Bordetella avium* sp. nov. was proposed. Further molecular characterization of *B. avium* has confirmed its unique taxonomic position among species of the *Bordetella* and *Alcaligenes* genera (14, 58, 72, 96, 130, 142). Recently the complete genome of *B. avium* (strain 197N) was elucidated (114). The genome is approximately 3.73 Mb in length and has an overall similarity of 97% (nucleotides) and 75% (proteins) with *B. pertusis, B. parapertusis*, and *B. bronchiseptica*. Almost a third of the predicted proteins for *B. avium* do not have an orthologue in *B. bronchiseptica* verifying that *B. avium* is the most distant member of the *Bordetella* genus.

Morphology and Staining

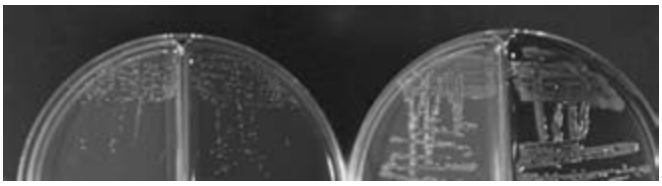
Bordetella avium is a gram-negative, nonfermentative, motile, strictly aerobic bacillus (71, 78) (see Table 19.5). Filamentous forms have been observed following the growth of *B. avium* in broth media high in nutrients (33).

Growth Requirements

It grows readily on MacConkey, Bordet-Gengou, veal infusion, trypticase soy blood agar, brain heart infusion (BHI), and many other solid media (4) but not on minimal essential medium (71). Trypticase soy and BHI broth support optimal growth when aeration is provided by agitation (9). Leyh *et al.* (83) have developed a defined minimal medium for growth of *B. avium* and detection of auxotrophic mutants.

Table 19.5. Physical properties of *Bordetella avium*.

Characteristics	References
Gram-negative rod (0.4–0.5 mm 3 1–2 mm)	78, 121
Strict aerobe	78, 121
Motile	77, 121
Capsulated	77, 121
Fimbriated (2 mm diameter)	69
Colonies, 0.2–1 mm at 24 hours; round, glistening	58, 78
Convex (some strains dissociate to larger colonies)	
Hemagglutination of guinea pig erythrocytes	47, 71
Erythrocytes of other species	58, 110
Growth temperature, optimal at 35°C, killed at 45°C	9
Generation time, 35–40 minutes at 35°C	9
Strict tropism for ciliated epitehlium	7, 49
Toxins	
Dermonecrotic (heat labile) toxin	42, 107, 108
Heat stable toxin	124
Osteotoxin	45
Tracheal cytotoxin	45
Guanine 1 cytosine composition of DNA, 61.6–62.6 mol%	78



19.21. Colonies of *Bordetella avium* strain 002 (left) and *B. hinzii* strain 128 (right) grown on MacConkey and blood agar, 48 hours at 37°C.

Colony Morphology

Most strains of *B. avium* produce small, compact, translucent, pearllike colonies (type I) with entire edges and glistening surfaces (78). Type 1 colonies are typically 0.2–1 mm in diameter after 24 hours of incubation and 1–2 mm in diameter after 48 hours of incubation. Many isolates develop a slightly raised brown-tinged center when grown 48 hours on MacConkey agar (Fig. 19.21). A small percentage of strains dissociate into a rough colony type with a dry appearance and a serrated irregular edge (74). Rough colonies were found to be nonpathogenic (74). A third colony type, characterized by a circular, convex colony with an entire edge, smooth surface, and larger size than type I colonies, has also been reported (58).

Biochemical Properties

Bordetella avium is a nonfermenting bacterium and is generally unreactive in biochemical tests. Biochemical properties of the organism are listed in Table 19.6. It is catalase and oxidase positive but the urease test and nitrate reduction test are negative.

Table 19.6. Biomechanical properties of *Bordetella avium*.

Biochemical test	Results	References
Oxidase (Kovac's reagent)	Positive	78, 121, 142
Catalase	Positive	58, 121, 142
Urease	Negative	55, 78, 121
Nitrate reduced to nitrite	Negative	57, 76, 110
Growth on MacConkey agar (lactose not fermented)	Positive	78, 121
Triple sugar iron agar	Alkaline slant, no change in butt	14, 71, 121
Alkalinize amides and organic salts (Greenwood's low peptone)	Several positive	14, 18, 19, 58

Susceptibility to Chemical and Physical Agents

Most commonly used disinfectants appear to kill *B. avium* when used according to manufacturers' recommendations. Survival of *B. avium* is prolonged by low temperatures, low humidities, and neutral pH (26). On simulated carrier materials, such as dust and feces from turkey houses, the organism survived 25–33 days at 10°C with a relative humidity 32–58%, whereas at 40°C with similar humidity the organism survived less than 2 days (26). Survival of the organism for at least 6 months in undisturbed damp litter has been reported (13). In BHI broth culture, bacteria are killed within 24 hours at 45°C (9). Survival may be greatly prolonged at 10°C on smooth surfaces such as glass or aluminum (26). Fumigation of an uncleaned room with methyl bromide effectively stopped transmission of the disease to day-old susceptible poults (116).

Resistance to streptomycin, sulfonamides, and tetracycline by some strains of *B. avium* is encoded on up to five plasmids ranging in size from 16–51.5 kb (31, 86); however, most strains are sensitive *in vitro* to a large number of antibacterials. Treatment of *B. avium*-infected turkeys with oxytetracycline administered parenterally or by aerosol results in either no effect or a transient reduction in bacterial numbers, even though the strain of *B. avium* was sensitive to oxytetracycline *in vitro* (38, 126, 140).

Antigenic Structure and Toxins

The antigenic structure of *B. avium* and related bacteria has been studied by agar gel precipitation, cross-agglutination, and Western immunoblotting (9, 52, 69, 71, 78). All evidence to date suggests that *B. avium* isolates from various sources are closely related antigenically (78). Using antisera produced in rabbits, Kersters *et al.* (78) identified 6 different surface antigens, 3 of which were cross-reactive among 3 strains of *B. avium.* In addition, they found 2 or 3 precipitation lines in common with *B. bronchiseptica.*

Further antigenic relatedness was demonstrated with *Alcaligenes denitrificans* and *Achromobacter xylosoxidans* (78). Jackwood *et al.* (71) have demonstrated antigenic cross-reactivity between *B. avium* and *B. hinzii* (see later discussion) bacteria. Using convalescent serum and tracheal washings in immunoblotting procedures, Hellwig and Arp (52) have shown that infected turkeys recognize at least 8 outer-membrane proteins of *B. avium.* These proteins range in size from about 14 to 116 kd. Leyh and Griffith (82) examined the outer-membrane protein profiles of 50 virulent *B. avium* isolates and found 2 major sarkosyl-insoluble proteins of 21 and 37 kD, and at least 13 other lesser proteins, using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). *B. avium* was found to have an electrophoresis profile distinctly different from those of both *B. hinzii* and *B. bronchiseptica.*

Varley and Carter (143) examined 7 *Bordetella* isolates from turkeys in the United Kingdom from the early 1980s using SDS-PAGE and compared those with known strains of *B. avium, B. bronchiseptica*, and *Alcaligenes faecalis.* All electrophoretic profiles were similar to both known *B. avium* and *A. faecalis* strains, indicating the usefulness of the procedure in distinguishing between *Bordetella* sp. but not for *A. faecalis.* Gentry-Weeks *et al.* (44) identified 5 outer-membrane proteins with molecular masses of 21, 38, 40, 43, and 48 kD being produced in *Escherichia coli* into which genes from *B. avium* had been cloned. Moore and Jackwood (95) produced monoclonal antibodies to a whole-cell *B. avium* preparation that recognized a 41-kD protein. These monoclonal antibodies were used to inhibit *B. avium* guinea pig red blood cell hemagglutination and could also be used to demonstrate the binding of the 41-kD protein to guinea pig erythrocytes. Hemagglutination was also inhibited following treatment of whole-cell *B. avium* with proteinase K indicating that part of the hemagglutinin is on the outside of the cell and with periodic acid, which cleaves carbohydrates from protein. This strongly suggests that the *B. avium* hemagglutinin is a carbohydrate closely associated with the 41-kD surface protein.

In a related study, Arp *et al.* (11) demonstrated complete inhibition of guinea pig red blood cell hemagglutination by *B. avium* using the gangliosides GD_{1a} and GT_{1b} and partial inhibition of hemagglutination using *N*-acetylneuraminic acid. When these same compounds, along with bovine submaxillary mucin and horseshoe crab lectin, were used to treat *B. avium,* adherence to the tracheal mucosa in turkeys was inhibited *in vivo*. The authors speculated that these compounds may be chemically related to the receptors for *B. avium* on the tracheal mucosa.

Five toxins are associated with *B. avium:* a heat-labile toxin (107), a dermonecrotic toxin (42), a tracheal cytotoxin (42), a heat-stable toxin (124), and an osteotoxin (45). Toxins of *B. avium* are considered to be virulence factors, and their activities are described in that section.

Strain Classification

Antigenicity

Studies of *B. avium* from various sources have revealed a great similarity in electrophoretic patterns of outer-membrane proteins (53, 78, 143). Furthermore, antigenic profiles examined by cross-agglutination, agar-gel precipitation, and Western immunoblotting showed little variation among *B. avium* strains (52, 78). *Bordetella avium* shares several cross-reactive antigens with *B. hinzii* and other *Bordetella* species (52, 71).

Genetic and Molecular

Despite the apparent genetic and molecular similarity among *B. avium* strains, differences have been noted in toxin production (20, 107, 124), adherence to tracheal mucosa (10), plasmid profiles (73, 125), antibiotic sensitivity (73), pathogenicity (54, 112), and colony morphology (74, 78). Recently, restriction enzyme analysis and ribotyping were used to characterize isolates of *B. avium*, and 12 different fingerprint profiles were observed (111). A *Hinf*I/*Dde*I restriction enzyme analysis was found to be useful as an epidemiological tool (104). In addition, those profiles were clearly distinguishable from other species of *Bordetella.*

Pathogenicity

Differences in pathogenicity have been reported among *B. avium* strains (110, 112). Differences in pathogenicity, associated with colony morphology and hemagglutination, led to categorization of isolates into various groups or types (14, 71, 110). Continuing study of the molecular characteristics of *B. avium* and related bacteria has identified several differentiating features of *B. avium* (Table 19.7). Strains previously referred to as group 1 (110) and type 1 (71) should now be called *B. avium.* Strains referred to as "*B. avium*-like," earlier (72), were assigned to a new species designated *B. hinzii* and will be reserved for nonpathogenic bacteria closely related to *B. avium* (141).

Table 19.7. Differentiation of *Bordetella avium* and *B. hinzii*.

Characteristics	*B. avium*	*B. hinzii*
Pathogenicity	Postive	Negative
In vivo adhesion[a]	Positive	Negative
Hemagglutination[b]	Positive	Negative
Growth on minimal essential medium agar (65)	Negative	Positive
Growth in 6.5% NaCl broth (65)	Few positive	Most positive
Other distinguishing features:		
Outer membrane profiles on SDS-PAGE[c] (53, 71)		
Cellular fatty acid analyses (72, 96)		
Alkalinization of amides and organic acids (14, 17)		

[a] Adhesion to turkey tracheal mucosa (5, 10).
[b] Hemagglutination of guinea pig erythrocytes (71) may be weak or inconsistent with some strains of organisms grown in liquid medium.
[c] SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrohoresis.

Virulence Factors

Major virulence factors of *B. avium* can be divided into those involved in either adhesion, local mucosal injury, or systemic effects. Adhesion to cilia of respiratory epithelium is a consistent trait of *B. avium* and other species of *Bordetella.* The surface structures or molecules of *B. avium* responsible for adhesion have been reported (11, 95), and the fimbriae (pili) and hemagglutinin may have roles (10, 68). Although fimbriae have been suggested as possible adhesive factors of *B. avium* (68), morphologically similar fimbriae are also common on adhesion-defective mutants and *B. avium*-like bacteria (53, 68). Hemagglutination (HA) of guinea pig erythrocytes correlates closely with virulence (42, 71) but appears to be unrelated to fimbriae (68). Two transposon-induced mutants selected for loss of HA activity had reduced adherence *in vivo* (10). Reversion of one mutant to HA-positive status resulted in the reconstitution of adherence.

Temple *et al.* (134), working with a hemagglutination-negative mutant of *B. avium*, reported that no clinical signs of disease were observed when given to 1-day-old or 1-week-old turkeys. As with other *Bordetella* species, it seems likely that more than one surface molecule is responsible for adhesion to cilia. Spears *et al.* (129), working with lipopolysaccharide (LPS) mutants of *B. avium*, showed that mutations in the *wlbA* and *wlbL* genes, two of the 12 genes associated with LPS biosynthesis, induced a clumped-growth phenotype and decreased tracheal colonization and serum resistance. A recent study showed that the core region and possibly the O antigen were associated with that phenotype (115).

Several local effects have been attributed to toxins of *B. avium.* An acute cytotoxic and ciliostatic effect of *B. avium* on turkey tracheal organ cultures was reported by Gray *et al.* (48, 50) and others (87). Rimler (107) described a heat-labile toxin capable of killing mice and young turkeys. The toxin was later shown to produce necrotic and hemorrhagic lesions in the skin of turkeys and guinea pigs after intradermal injection and similar lesions in the liver and pancreas of turkeys following intraperitoneal injection (106, 108). Recent work has shown that *B. avium* produces a dermonecrotic toxin with physical, antigenic, and biologic properties comparable to those reported for the heat-labile toxin (42). The dermonecrotic toxin is a cell-associated, 155-kD protein with biologic activity comparable to dermonecrotic toxins of *B. pertussis* and *B. bronchiseptica* (42). The toxin appears not to be responsible for ciliostasis (108) or local epithelial damage (136). Gentry-Weeks *et al.* (43) produced spontaneous-phase variants of *B. avium* that lacked dermonecrotic toxin and four outer-membrane proteins when grown in media containing nicotinic acid and $MgSO_4$. These variants had a different colony morphology but retained the ability to agglutinate guinea pig red blood cells. Passage in susceptible turkeys caused these variants to revert to the wild type. A role for the dermonecrotic toxin has been established in the pathogenesis of bordetellosis in turkeys by Temple *et al.* (134), who reported that a dermonecrotic toxin-negative mutant was nonpathogenic when given to 1-day-old or 1-week-old turkeys.

Another toxin of *B. avium* implicated in local mucosal injury is the tracheal cytotoxin (TCT) isolated by Gentry-Weeks *et al.*

(42). The TCT of *B. pertussis,* which is chemically identical to that produced by *B. avium,* has been shown to specifically damage ciliated epithelial cells leading to a loss of epithelium and poor clearance of mucus (47). The TCT of *B. avium* is an anhydropeptidoglycan monomer with a mass of 921 kD. Whether TCT is the mediator of cytotoxic activity reported earlier by Gray *et al.* (48, 50) is unclear.

Simmons *et al.* (124) have identified a *B. avium* heat-stable toxin capable of causing diarrhea and death in mice inoculated intraperitoneally; however, no evidence shows that the toxin produces adverse effects in poultry. None of 18 *B. avium* strains from Australia had the mouse-lethal toxin that was found in several reference strains from other turkey-producing areas in the world (20). An osteotoxin recently was found to be associated with *B. avium.* It has been identified as beta cystathionase and is lethal to MC3T3-E1 osteogenic cells, fetal bovine trabecular cells, UMR106-01 (BSP) rat osteosarcoma cells, and embryonic bovine tracheal cells (45). This toxin might be responsible for the cartilage lesions that lead to tracheal softening and collapse. Examination of several *B. avium* strains for the production of extracytoplasmic adenylate cyclase (42, 108) or pertussis toxin (42) failed to detect either one by immunologic and functional assays. Earlier studies by Simmons *et al.* (123) suggested that *B. avium* produces a histamine-sensitizing factor similar to that produced by other *Bordetella* species.

Virulence factors in *Bordetella* spp. have been shown to be coordinately regulated at the genetic level by the *bvg* (bordetella virulence gene) locus. Expression of virulence factors can be modulated by environmental conditions including temperature, sulfate ions, and nicotinic acid. This phenomenon is termed antigenic variation and has been reported in *B. avium* (74). In addition, genetic evidence for a *bvg* locus in *B. avium* has been reported (43,114). In a study to detect virulence genes of *B. pertussis* by Southern hybridization, it was determined that the *bvgS* gene was present in *B. avium* but the *bvgA* was not (43). Since that report, the *B. avium bvg* homolog has been cloned and sequenced, which showed a frame shift in the poly (C) tract suggesting a different phase variation mechanism in *B. avium* from other *Bordetella* (27, 114).

Acquisition of iron (Fe) from the host, an essential characteristic for most bacteria, was shown to be important for colonization and proliferation of *B. avium* (98). The *B. avium* Fe utilization locus consists of a putative outer-membrane heme receptor (BhuR) that mediates acquisition of Fe from hemin and hemoproteins, and six other accessory genes (rhuIR and bluSTUV). Expression of BhuR is regulated by sigma factor rhuI which is likely expressed in a Fe uptake regulator (Fur) dependent manor in response to environmentally available Fe (79, 80, 81).

A number of systemic pathophysiologic effects have been attributed to *B. avium* infection. These effects include elevation of serum corticosterone (93), enhanced leukocyte migration (90), altered electrocardiograms (145), reduced body temperature (34), reduced levels of monoamines in brain and lymphoid tissues (35, 36), reduced levels of liver tryptophan 2,3-dioxygenase (144), and reduced thyroid hormones in conjunction with fasting (37). Beginning with the original recognition of turkey rhinotracheitis in North Carolina, reports from flock service people have suggested defective immune function in affected poults (120). Vaccination of these poults with live vaccines resulted in unexpected deaths. This background, along with the observation of reduced bursa size in some poults with rhinotracheitis (118), led to a series of experiments to determine effects of *B. avium* infection on immune function. Initial studies in poults infected with *B. avium* (120) found a decreased lymphocyte blastogenesis response to concanavalin A and a depletion of thymic lymphocytes. Subsequent studies of cell-mediated immunity in *B. avium*-infected poults showed the reverse effect with enhanced graft-versus-host and delayed hypersensitivity responses (91, 92), both measures of cell-mediated immunity.

Pathobiology and Epidemiology

Incidence and Distribution

Bordetellosis is an important disease in major turkey-producing regions of the United States, Canada (23), Australia (18), and Germany (56). The etiology of turkey rhinotracheitis in Great Britain, France, Israel, and South Africa, however, frequently may include viruses and other bacteria in addition to *B. avium* (51, 85). Hopkins *et al.* (63) detected *B. avium* antibodies by enzyme-linked immunosorbent assay (ELISA) in 42 of 44 wild turkeys being translocated in Arkansas. Thus, *B. avium* may be a significant problem in wild turkeys, or it may be that wild turkeys act as a reservoir for the infection. McBride *et al.* (89) surveyed three backyard turkey flocks located within 1 mile of commercial turkey farms in California and found all turkeys sampled at each location to be seropositive to *B. avium* when tested by microagglutination. A survey between 1998 and 2000 found *B. avium* antibodies in sera from 100 individuals representing 41 different species of wild and domestic birds (103). In addition, Raffel *et al.* (103) isolated *B. avium* from mallards (*Anas platyrhynchos*), wild turkey (*Meleagris gallopavo*), and Canada goose (*Branta canadenisis*). Hollamby *et al.*(61) detected antibodies against *B. avium* in peafowl (*Pavo cristatus*).

Natural and Experimental Hosts

The natural host of *B. avium* is the turkey, although isolations of *B. avium* have also been made from chickens and other avian species (58, 122). Strains of *B. avium* isolated from avian species other than turkeys are pathogenic for day-old turkeys (58). A study of the prevalence of *B. avium* in North Carolina broiler flocks during the winter months revealed a 62% infection rate (16). Furthermore, a higher isolation rate was found from flocks with respiratory disease. Attempts to reproduce rhinotracheitis experimentally in chickens revealed that only 2 of 8 *B. avium* strains colonized the trachea and produced disease (15); however, a later study (14) suggested the isolations from chickens may have included both *B. avium* and *B. hinzii* bacteria. Finally, it was reported that *B. avium* appears to be an opportunistic pathogen in chickens (66). Prior exposure to an upper-respiratory disease vaccine, such as infectious bronchitis virus or Newcastle disease virus, was necessary to induce clinical signs in leghorn chickens.

Turkey and chicken strains of *B. avium* are similar (78), and

cross-infection can occur between the species (122). Bordetellosis in chickens tends to be less severe than in turkeys (94, 122). A strain of *B. avium,* pathogenic for turkeys and Japanese quail, failed to produce clinical disease in guinea pigs, hamsters, and mice (88). Naturally occurring infection with *B. avium* typically is recognized in turkeys 2–6 weeks old (23, 56, 101), although older turkeys and breeder flocks may also develop clinical disease (75, 77). Experimental inoculation of poults more than 1–2 weeks old frequently results in colonization but with only mild disease.

Transmission and Carriers

Bordetellosis is highly contagious. The disease is readily transmitted to susceptible poults through close contact with infected poults or through exposure to litter or water contaminated by infected poults (116). Infection is not transmitted between adjacent cages, thus providing evidence against aerosol transmission (116). Litter contaminated by a flock infected with *B. avium* is likely to remain infective for 1–6 months (13, 26). Although a carrier state has not been demonstrated in turkeys recovered from bordetellosis, the possibility seems likely.



19.22. Clinical appearance of a poult with bordetellosis. Open-mouth breathing, dark stains around eye and nostril, and foamy exudate at the medial canthus of the eye.

Incubation Period

The incubation period is 7–10 days when susceptible poults are exposed to infected poults by close direct contact (116). Intranasal or intraocular inoculation of day-old poults with 10^5 to 10^7 colony-forming units of *B. avium* results in clinical signs (nasal exudate) of bordetellosis in 4–6 days (6, 49, 112).

Clinical Signs

An abrupt onset of sneezing (snick) in a high percentage of 2–6-week-old turkeys over the course of a week is suggestive of bordetellosis. Older turkeys may also develop a dry cough (77). A clear nasal discharge can be expressed by placing gentle pressure over the bridge of the beak between the nostrils. During the first 2 weeks of disease, the nares and feathers of the head and wings become crusted with wet, tenacious, brownish exudate (Fig. 19.22), and some birds develop submaxillary edema. Mouth breathing, dyspnea, and altered vocalization in the second week of clinical signs result when the nasal cavity and upper trachea become partially occluded with mucoid exudate. Tracheal softening can be palpated through the skin of the neck in some birds beginning in the second week of disease. Behavioral changes include reduced activity, huddling, and decreased consumption of feed and water. Concurrent infections and poor weight gains contribute to poor flock performance and numerous birds with stunted growth (9). Signs of disease begin to subside after a course of 2–4 weeks (49, 101, 112, 139).

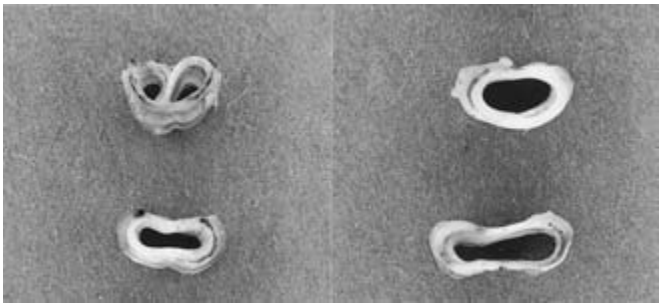
Morbidity and Mortality

Bordetellosis in turkeys is typically characterized by high morbidity and low mortality. In turkeys 2–6 weeks of age, morbidity reaches 80–100% (112), whereas the mortality rate is less than 10%. Infection of a breeder flock with *B. avium* resulted in only 20% morbidity with no mortality (77). High mortality rates (>40%) in young turkeys frequently are associated with concurrent isolation of *Escherichia coli* (23, 112). Experimental studies of concurrent *B. avium* and *E. coli* infections in 2–4-week-old turkeys revealed defective clearance of *E. coli* from tracheas (39, 137) and increased severity of airsacculitis attributable to *E. coli* (138). Adverse environmental temperatures (9), high humidity (127), poor air quality, and concurrent respiratory pathogens may increase mortality rates (112). Cook *et al.* (29) studied the interaction of turkey rhinotracheitis virus (TRTV), a pneumovirus, with *B. avium* and a *Pasteurella*-like organism in turkeys. When the TRTV was administered alone, the virus could be isolated from only the trachea, but when given in combination with the bacteria, it was capable of invasion and could be isolated from the heart, liver, spleen, kidney, and cecal tonsils. Hinz *et al.* (59) described an outbreak of *B. avium* in combination with *Chlamydia psittaci* in 6 different turkey flocks on a large multiple-age grow-out operation. Mortality in the affected flocks ranged from 7–20%, and the high mortality was attributed to secondary infections from *Klebsiella pneumoniae, E. coli,* and *Pseudomonas fluorescenc.*

Pathology

Gross

Gross lesions are confined to the upper respiratory tract and vary with the duration of infection. Nasal and tracheal exudates change in character from serous initially to tenacious and mucoid during the course of disease. Tracheal lesions consisting of generalized softening and distortion of the cartilaginous rings, dorsal-ventral compression, and fibrinomucoid luminal exudate are highly suggestive of bordetellosis (6, 139). In isolated cases, there is severe infolding of the dorsal tracheal wall into the lumen immediately below the larynx (Fig. 19.23) (6, 140). In cross-section, tracheal rings appear to have thick walls and a dimin-



19.23. Cross-sections of a collapsed trachea from a poult with bordetellosis. Section on the top left, taken immediately below the larynx, has extreme dorsal-ventral infolding. Other sections were taken at 5 cm intervals along the trachea. (*Am J Vet Res*)

ished lumen. Distortion of tracheal cartilages persists at least 53 days postinfection (6). Accumulation of mucoid exudate in an area of tracheal infolding frequently leads to death by suffocation (6). Hyperemia of the nasal and tracheal mucosae and edema of interstitial tissues of the head and neck are apparent during the first 2 weeks of infection.

Microscopic

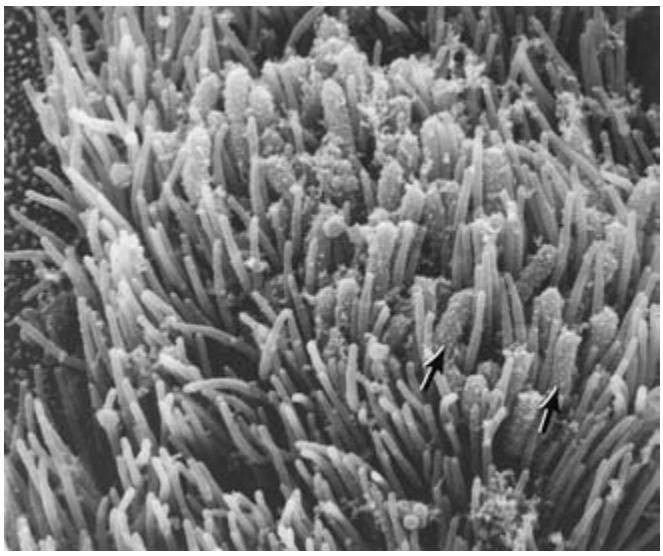
Cilia-associated bacterial colonies, progressive loss of ciliated epithelium, and depletion of mucus from goblet cells are distinctive characteristics of bordetellosis (6). Colonization of ciliated epithelium begins on the nasal mucosa, progresses down the trachea, and moves into primary bronchi within 7–10 days. Bacteria adhere specifically to cilia and have not been found attached to other cell types (7). As seen by scanning electron microscopy, surfaces of adherent bacteria are covered with numerous knoblike surface projections (Fig. 19.24). Colonized cells having increased eosinophilia of the apical cytoplasm may protrude slightly from the mucosa (140). Bacterial colonies (Fig. 19.25) are most apparent on the tracheal mucosa 1–2 weeks after onset of clinical signs, before loss of ciliated cells is extensive (6, 7).

Ultrastructural

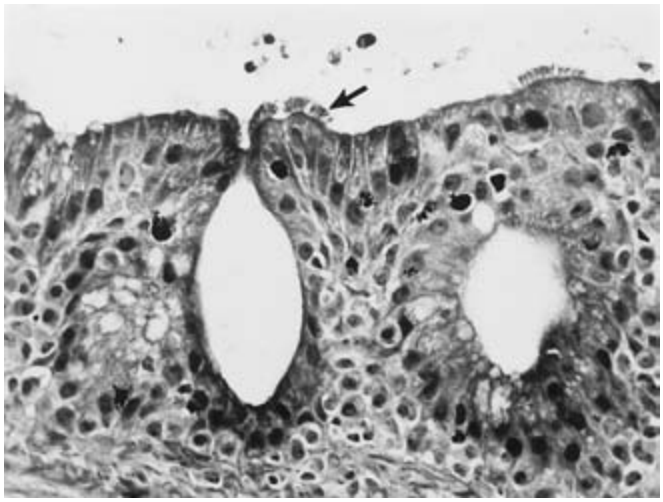
During the first 2 weeks of signs, ciliated tracheal epithelium is gradually lost and replaced by nonciliated cuboidal epithelium (Fig. 19.26). These immature hyperplastic cells have basophilic cytoplasm with variable numbers of small mucous granules (6, 140). Late in the disease, squamous metaplasia of tracheal epithelium may occur (Fig. 19.27). Linear, eosinophilic inclusions occur in the cytoplasm of tracheal epithelium during the first 3 weeks of disease (6, 7). Ultrastructurally, these inclusions are proteinaceous crystals composed of parallel filaments surrounded by membrane (7). During the third and fourth week of disease, the tracheal mucosa becomes distorted by numerous folds and mounds of dysplastic epithelium. Depending on the severity of the disease, the tracheal epithelium returns to normal 4–6 weeks after the onset of signs (6, 49), when *B. avium* can no longer be isolated.

Discharge of copious mucoid exudates from the upper respiratory tract is accompanied by depletion of mucus from isolated goblet cells and mucous glands along the mucosa (6, 140). Alveolar glands become cystic and lined by immature epithelium with small mucous granules (Fig. 19.25). Goblet cells remain largely depleted of mucous granules from the first through the third week of clinical disease.

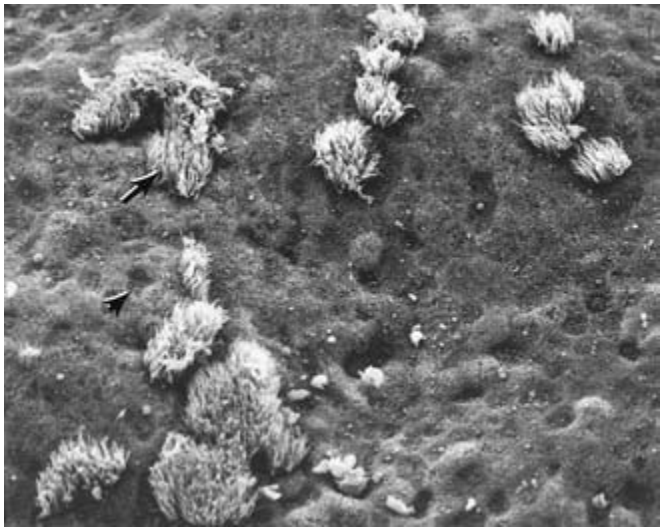
Cellular exudates in the tracheal lamina propria begin with multifocal infiltrates of heterophils and change to predominantly



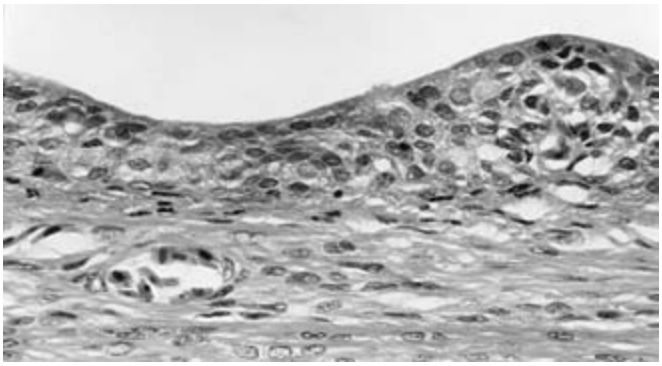
19.24. Numerous *Bordetella avium* bacteria (arrows) intimately associated with cilia of tracheal epithelial cells. The bacterial surfaces are covered with irregularly shaped, knoblike projections, which may contribute to adhesion.



19.25. Trachea from a poult infected 3 weeks previously with *Bordetella avium*. Characteristic lesions of brodetellosis include cilia-associated bacterial colonies (arrow), loss of ciliated epithelium, dilated mucous glands depleted of mucus, and interstitial infiltration of plasma cells and lymphocytes.



19.26. Loss of ciliated epithelium from the tracheal mucosal surface. Isolated clumps of ciliated cells (arrow) and dark pits left where ciliated cells have sloughed (arrowhead).



19.27. Squamous metaplasia of tracheal epithelium occurs in some poults late in the course of bordertellosis.

lymphocytes and plasma cells as clinical signs subside (6, 49). In the third through fifth week of disease, a diffuse increase in mucosal plasma cells is accompanied by multifocal lymphoid nodules in the submucosa. Mucosal surface exudates change from mucopurulent to fibrinopurulent after the first week of disease (7)

Pulmonary lesions are restricted to primary bronchi and bronchus-associated lymphoid tissue (138, 139). In contrast to the tracheal mucosa, the bronchial mucosa maintains a near normal appearance including ciliated columnar epithelium and goblet cells (139). Mild colonization of isolated ciliated cells by *B. avium* is accompanied by a mild infiltrate of heterophils. Bronchus-associated lymphoid tissue, normally found at the junction of primary and secondary bronchi, becomes grossly apparent, and lymphoid nodules protrude into the bronchial lumen (139). Other changes of lymphoid tissues include the depletion of cortical lymphocytes from the thymus during the early disease (120)

In summary, distinctive microscopic lesions of diagnostic value include cilia-associated bacterial colonies, cytoplasmic inclusions, cystic mucosal glands, and generalized loss of ciliated epithelium.

Pathogenesis of the Infectious Process

Initial adhesion of bacteria to ciliated cells of the oronasal mucosa leads to progressive colonization from the upper trachea to the primary bronchi over the next week. Expansion of the bacterial population along the respiratory mucosa stimulates acute inflammation and the release of mucus from goblet cells leading to sneezing, coughing, and nasal obstruction. Spread of infection against the flow of mucociliary clearance occurs as motile "swarmer" bacteria break free from microcolonies and move within the layer of mucin to other ciliated cells. Apparently, tracheal mucus does not contain receptor analogs to impede the spread of the bacteria. During the next week, many of the cells colonized by *B. avium* slough into the tracheal lumen, leaving large surfaces devoid of cilia.

How *B. avium* damages the tracheal mucosa and cartilage remains unknown, but the tracheal cytotoxin may be involved. The formation of cytoplasmic protein crystals and delayed restitution of normal mucosa are suggestive of a toxin that alters cell growth and differentiation. The molecular basis for softening and the collapse of tracheal rings may result from abnormal connective tissue metabolism leading to qualitative and quantitative changes in collagen and elastin (144).

As ciliated cells are progressively lost, the flow of mucus and exudates becomes sluggish, particularly in the upper trachea and nasal cavity. Obstruction of nasolacrimal ducts causes foamy ocular exudate to accumulate at the medial canthus of the eye. Signs of bordetellosis result from local and systemic products of the inflammatory response, soluble bacterial toxins, and physical obstruction of large air passages.

Within a week of the onset of clinical signs, local and systemic immune responses develop to *B. avium* antigens. Antibody transported from serum and antibody produced by submucosal plasma cells accumulates in respiratory secretions. Local antibody interacts with free "swarmer" *B. avium* cells to inhibit their motility and to prevent adhesion to other ciliated cells. Colonies of bacteria among the cilia are largely protected from host defenses; however, numerous bacteria are shed along with colonized epithelial cells. The bacterial population diminishes over the next several weeks as colonized cells are lost, and newly formed ciliated cells are protected from colonization by antibody.

Some convalescent birds are probably slow to clear all *B. avium* from their respiratory tissues and serve as a source of infection for susceptible flocks. As mucosal immunity wanes over the next 4–8 weeks, any residual population of *B. avium* in the nasal cavity or sinuses can again expand to produce clinical infection or be transmitted to susceptible birds.

Immunity

Active

An active immune response is generated in most turkeys inoculated with live *B. avium* or various bacterins. The serum antibody

response to a temperature-sensitive mutant of *B. avium* is variable, depending on vaccination dosage, turkey age, and environmental factors affecting colonization (9, 25, 54, 60, 64, 67, 75). Recent studies have suggested that poults less than 3 weeks of age respond poorly to *B. avium* vaccines (60, 67).

Most turkeys develop a humoral immune response to infection with *B. avium* (6, 65, 133). Serum antibodies, detected by microtiter agglutination, appear within 2 weeks after experimental exposure to *B. avium* and reach peak levels by 3–4 weeks postexposure (6, 65). The period of peak antibody titer is followed within 1 week by resolution of clinical disease and a decline in bacterial numbers in the trachea (6). This, combined with evidence for maternal immunity, suggests an important role for humoral immunity in the prevention and recovery from infection (13, 57).

Infection with *B. avium* has been recognized as potentially immunosuppressive since Simmons *et al.* (120) reported thymic lesions and suppression of lymphocyte blastogenesis. Although subsequent tests have revealed no evidence for defects in cellular immunity (90, 91, 92), infection with *B. avium* apparently interferes with immunity to live *Pasteurella multocida* and hemorrhagic enteritis vaccines (109, 120). Reduced monoamine concentrations in brain and lymphoid tissues and elevated serum corticosterone have been recorded in turkeys infected with *B. avium* (35, 36, 93). Although such hormonal changes are probably not unique to bordetellosis, they may help explain the immunosuppression seen in the field.

Passive

Neighbor *et al.* (99) evaluated maternal antibody in poults from immunized and nonimmunized hens. Resistance to clinical disease and gross lesions was greatest in poults with maternal antibody, as measured by ELISA. Convalescent serum and tracheal secretions from turkeys infected with *B. avium* inhibit adherence of the bacteria to the tracheal mucosa in turkeys (8). Moreover, adherence of *B. avium* is inhibited whether convalescent serum is administered locally or parenterally. The passive administration of convalescent serum is believed to mimic many aspects of maternal immunity. Suresh *et al.* (133) evaluated antibody levels in serum, tracheal washings, and lacrimal secretions from turkeys experimentally infected with *B. avium.* Turkeys were sacrificed at weekly intervals through 8 weeks postinoculation. Maternal antibody was undetectable by 3 weeks of age, and the appearance of serum and mucosal antibody was associated with clearance of *B. avium* from the trachea. Using serum and tracheal secretions collected from turkeys during a 4-week course of infection, at least 8 *B. avium* surface proteins were identified using Western immunoblots (52).

Diagnosis

The diagnosis of bordetellosis is currently based on clinical signs and lesions, isolation of *B. avium* from the respiratory tract, a positive serologic test, or some combination of these. Additional diagnostic techniques that have been developed include a monoclonal antibody-based latex bead agglutination test (132), an indirect fluorescent antibody staining technique using a monoclonal antibody (131), a capillary gas chromatography assay for cellular carbohydrates using per-acetylated aldonitriles and *O*-methyloximes (97), and a polymerase chain reaction technique (105, 113).

Isolation and Identification of Causative Agent

Bacterial isolation is accomplished on MacConkey agar inoculated with a swab sample from the tracheal mucosa. Samples collected from the choanal opening and nostril, or by passing a swab into the trachea through the larynx, commonly yield large numbers of nonpathogenic bacteria (119, 128). When turkeys are available for necropsy examination, swab samples should be collected aseptically through an opening in the midcervical trachea. After 24 hours of incubation on MacConkey agar, colonies of *B. avium* are clear and pin-point in size. Although most contaminating bacteria form large mucoid colonies (often lactose fermenters), which mask *B. avium* in the more concentrated streak pattern, the minute colonies of *B. avium* may be recognized in the more diluted streak pattern. By incubating culture plates up to 48 hours, *B. avium* colonies are more easily recognized and may develop a brownish raised center (Fig. 19.21). Early in the course of infection, pure cultures can be obtained from the trachea, but in later stages, *E. coli* and other opportunistic bacteria may be isolated (112). Physical and biochemical characteristics that serve to distinguish *B. avium* from closely related bacteria are presented in Tables 19.6 and 19.7.

Serology

Serologic testing has proven to be useful experimentally and in the natural disease for detection of serum antibodies to *B. avium.* Jackwood and Saif (65) developed a microagglutination test (MAT) using a killed, neotetrazolium-chloride-stained *B. avium* antigen in a microtiter system. The MAT has been shown to correlate well with bacterial isolation. It seems likely that serologic tests remain positive for a period after *B. avium* can no longer be cultured. In a field study by Slavik *et al.* (128), flocks with a history of respiratory disease were more commonly positive serologically for *B. avium*, even though bacteria were not isolated. In experimentally infected poults, antibody is detectable by the MAT from 2 weeks postinoculation (PI) until at least 5–7 weeks PI (6, 9, 65). Peak titers occur at about 3–4 weeks PI. For each of the preceding tests, agglutination occurs at serum dilutions of from 1:320 to 1:512 (6, 65). Use of heterologous *B. avium* antigen has little effect on agglutination titers (9).

Hopkins *et al.* (62) developed an ELISA for detection of serum antibodies to *B. avium* using a whole-bacteria antigen, a 1:200 serum dilution, and a 1:3200 dilution of commercially available anti-turkey IgG conjugate. Serologic results obtained with ELISA correlate well with those from the MAT, but ELISA is more sensitive for detection of maternal antibody in day-old poults (62). Although *B. avium* has antigens in common with closely related *B. hinzii* bacteria, no evidence indicates that these related bacteria cause a positive serologic reaction to *B. avium* in nature. Several different variations of ELISA procedures have been developed for *B. avium* (12, 22, 99, 133, 135). The varia-

tions include a dot-immunobinding assay (135) and a particle concentration fluorescence immunoassay (22). All of these detect maternal antibody and are reproducible and sensitive. Recently a commercially available ELISA kit has become available, and it has proven to be very useful (84).

Differential Diagnosis

Bordetellosis must be differentiated from other primary and secondary causes of rhinotracheitis. Mycoplasmosis, chlamydiosis, and respiratory cryptosporidiosis may mimic or contribute to many of the clinical signs of bordetellosis (2, 59, 77, 85). Of the viral agents, Newcastle disease virus, Yucaipa virus, adenovirus, influenza virus, and pneumovirus should be considered (28, 85). Although *B. avium* alone can produce all of the clinical signs and lesions of bordetellosis in the natural disease, *B. avium* is more frequently accompanied by Newcastle disease, *Mycoplasma* spp., and opportunistic bacteria such as *E. coli.*

Currently, the greatest diagnostic challenge is to differentiate *B. avium* from *B. hinzii* bacteria in primary cultures. Distinguishing characteristics of these closely related bacteria are presented in Table 19.7; however pathogenicity testing in day-old poults is definitive. Intranasal inoculation of day-old poults with a 24-hour broth culture of *B. avium* is expected to produce clinical disease and nasal discharge in susceptible poults within 3–5 days.

Intervention Strategies

Management Procedures

Bordetella avium is highly contagious by direct contact and through contamination of water, feed, and litter. Strict biosecurity measures are required to prevent infection of clean flocks, and rigorous cleanup procedures are required to eliminate the organism from contaminated premises. A minimal cleanup procedure for contaminated premises should include complete removal of litter, thorough washing of all surfaces, disinfection of watering systems and feeders, and the application of a disinfectant followed by either formaldehyde fumigation or by the application of a dilute formaldehyde solution to all surfaces. *Bordetella avium* is tracked easily from one facility to another, so the use of disinfectant foot baths, clean outer clothing, and even a required shower between visits to different houses and locations is essential. Because the severity of bordetellosis is exacerbated by adverse environmental and infectious factors, attempts should be made to optimize temperature, humidity, and air quality while avoiding or delaying the use of live attenuated vaccines.

Vaccination

Vaccines available commercially for the prevention of bordetellosis in turkeys include a live temperature-sensitive (ts) mutant of *B. avium* (Art Vax^{TM}, Schering-Plough Animal Health, Union, NJ) and a whole-cell bacterin ADJUVAC-ART, which used to be available from Sanofi Animal Health, Inc., Overland Park, KS. The live ts-mutant vaccine was induced by nitrosoguanidine mutation of a virulent *B. avium* isolate obtained from North Carolina (24). Original studies indicated that the ts mutant colonized the nasal mucosa and induced moderate levels of serum antibodies (24). Although subsequent use of the vaccine in Utah indicated substantial protection (25, 75), other controlled experiments indicated only moderate reduction in lesion severity or delayed the onset of clinical disease (54, 60, 64, 67). The ts mutant has the capacity to adhere to respiratory epithelium, but its slow growth rate may critically limit its ability to colonize and induce protective immunity (9, 10). Use of the ts-mutant vaccine, according to label directions in day-old poults, failed to prevent infection and disease; however, use of the vaccine in poults 3 weeks of age and older prevented disease but not infection (60, 67). Concern exists that turkeys less than 3 weeks of age may be unable to respond adequately to *B. avium* antigens or are unable to mount an adequate local immune response. Glunder *et al.* (46) inoculated killed *B. avium* with adjuvant into 1, 7, 10, 14, and 21 day old poults and found that regardless of age at vaccination, serum antibodies were first detected at 28 days of age.

Houghten *et al.* (64) compared a spray method of vaccination with the method recommended by the manufacturer for the ts-mutant vaccine. Turkeys were immunized at 2 days of age, using a spray cabinet, followed 14 days later with another coarse spray exposure to the ts-mutant vaccine. Another group of turkeys was similarly immunized by eyedrop exposure followed 14 days later by oral exposure. The spray and eyedrop/oral methods of immunization were equally effective in reducing the severity of tracheal lesions, but neither method prevented infection of the trachea by virulent challenge strains.

Several studies have indicated that breeder hen vaccination may be useful for prevention of bordetellosis in progeny poults (13, 57, 99). Vaccination of breeder hens with either heat-killed (57) or formalin-killed (13) adjuvanted bacterins delayed the onset and severity of clinical disease in challenge-exposed poults. Passive immunization of 3-week-old poults with convalescent serum reduces adherence of *B. avium* to the tracheal mucosa in a dose- and time-dependent manner (8). Taken in total, these studies suggest that maternal antibody of the IgG class may confer temporary immunity to newly hatched poults. Additionally, vaccination of poults with purified pilus preparations and adjuvanted bacterins results in significant protection against *B. avium* colonization and clinical disease (1).

Because *B. avium* and *B. hinzii* bacteria are antigenically related, Jackwood and Saif (69) designed experiments to determine whether poults infected with nonpathogenic *B. hinzii* bacteria would develop immunity to *B. avium.* The *B. hinzii* bacteria failed to persist for a significant period in the respiratory tract and failed to induce either a serologic response or protection to *B. avium* challenge. Development of improved vaccines for bordetellosis requires better characterization of protective antigens of *B. avium* and an understanding of the turkey's immune response to them.

Treatment

Treatment of bordetellosis with antibiotics administered in the water, by injection, or by aerosol has produced minimal clinical improvement in most cases. Treatment of an infected breeder flock with 1.8 g tetracycline-HCl and 2×10^6 IU potassium peni-

cillin-G per gallon of drinking water for 3 days produced clinical improvement within 24 hours (77). Treatment of bordetellosis in young turkeys with an aerosol of oxytetracycline-HCl reduced mortality associated with subsequent Newcastle disease vaccination compared with untreated flocks (38). Although these clinical testimonials suggest a favorable response to treatment, it remains unclear whether clinical improvement results from antibacterial effects against *B. avium* or to secondary pathogens such as *E. coli.*

In a group of experimentally infected poults, parenteral administration of long-acting oxytetracycline had no apparent effect on *B. avium* infection (126). Treatment of experimental bordetellosis with oxytetracycline-HCl administered by aerosol caused a transient reduction of bacterial numbers in the trachea and a delay in clinical signs and lesion development (140). However, by 4 days after treatment, bacterial numbers and disease severity were similar between treated and nontreated groups (140).

Yersin *et al.* (146) were able to demonstrate up to a 40% reduction in the loss of cilia following the treatment of *B. avium*-infected turkeys with niacin added to the drinking water at 70 mg/L. Niacin treatment also reduced clinical signs, increased body weight, and reduced adherence of bacteria to the tracheal epithelium when treated turkeys were compared with untreated infected turkeys. The authors speculate that the mechanism for this therapeutic effect may be the result of the inhibition of glucocorticoid-induced DNA strand breakage and subsequent ADP-ribosylation of nuclear DNA. This action would allow for the continued protein synthesis necessary to maintain ATP-mediated functions in the cilia of the trachea. Pardue and Luginbuhl (102) observed that administration of 0.016% oxy-halogen formulation in the drinking water at 4, 7, 10, 14, and 17 days of age reduced many of the signs associated with bordetellosis in turkeys.

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Chapter 20

Infectious Coryza and Related Bacterial Infections

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Introduction

Infectious coryza (IC) is an acute respiratory disease of chickens caused by the bacterium *Avibacterium paragallinarum*, once known as *Haemophilus paragallinarum* (13). The taxonomic changes that resulted in the recognition of *Av. paragallinarum* also allocated the bacterium once known as *Pasteurella gallinarum* to the same genus as *Av. gallinarum* (13). Hence, this chapter has integrated our current knowledge on both bacterial species, *Av. paragallinarum* and *Av. gallinarum,* and the diseases associated with both bacteria.

The clinical syndrome of infectious coryza has been described in the early literature as roup, contagious or infectious catarrh, cold, and uncomplicated coryza (156). The disease was named infectious coryza because it was infectious and affected primarily the nasal passages (6). No specific syndrome name has been allocated to the disease conditions associated with *Av. gallinarum*.

Economic Significance

The greatest economic losses associated with infectious coryza result from poor growth performance in growing birds and marked reduction (10–40%) in egg production in layers. The disease can have a much greater impact than the relatively simple scenario described above. As an example, an outbreak of the disease in older layer birds in California, which was not associated with any other pathogen, caused a total mortality of 48% and a drop in egg production from 75% to 15.7% over a 3-wk period (27).

Infectious coryza can have significant impact in meat chickens. In California, two cases of infectious coryza, one complicated by the presence of *Mycoplasma synoviae,* caused increased condemnations, mainly due to airsacculitis, that varied from 8% to 15% (52). In Alabama, an infectious coryza outbreak in broilers that was not complicated by any other disease agent, caused a condemnation rate of 69.8%, virtually all due to airsacculitis (70).

When infectious coryza occurs in chicken flocks in developing countries, the added presence of other pathogens and stress factors can result in disease outbreaks that are associated with greater economic losses than those reported in healthy flocks in developed countries. In China, outbreaks of infectious coryza have been associated with morbidities of 20% to 50% and mortalities of 5% to 20% (44). In Morocco, outbreaks on 10 layer farms caused egg drops that ranged from 17 to 41% and mortalities of 0.7 to 10% (95). A study of village chickens in Thailand has shown that the most common cause of death in chickens less than 2 months old and those more than six months old was infectious coryza (148). It was only in chickens that were between 2 and 6 months of age that other diseases such as Newcastle disease and fowl cholera killed more chickens than infectious coryza (148). Infectious coryza has also been reported in kampung (village) chickens in Indonesia (108, 142). Overall, there is considerable evidence that infectious coryza outbreaks can have a much greater impact in developing countries than in developed countries.

Outbreaks of disease associated with *Av. gallinarum* have not been commonly reported. Mortalities in chickens have been reported at 5–10% (28) and 10–34% (54). In turkeys, mortalities of 18–26% have been reported (8). In most cases of disease associated with *Av. gallinarum*, the possibility that other infectious agents such as viruses and mycoplasmas were involved in the overall disease complex exists.

Public Health Significance

There is no public health significance to *Av. paragallinarum*. There are three reports of *Av. gallinarum* being a possible cause of disease in humans (1, 2, 4). However, these reports lack definitive molecular or phylogenetic data and some have been suggested to be misidentifications (59). On the balance of the available evidence, *Av. gallinarum* does not appear to have public health significance.

History

As early as 1920, Beach (5) believed that infectious coryza was a distinct clinical entity. The etiologic agent eluded identification for a number of years, since the disease was often masked in mixed infections and with fowlpox in particular. In 1932, De Blieck (49) isolated the causative agent and named it *Bacillus hemoglobinophilus coryzae gallinarum.*

The first report to draw attention to organisms that appear to resemble the organism *Av. gallinarum* was Schneider (132). The description of the species *P. gallinarum* occurred in 1955 (64).

Etiology

Classification

Based on studies conducted during the 1930s, the causative agent of IC was classified as *H. gallinarum* because of its requirement

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for both X-(hemin) and V-(nicotinamide adenine dinucleotide—NAD) factors for growth (57, 131). In 1962, Page (104) reported that all isolates recovered from cases of IC required only the V-factor for growth. This led to the proposal and general acceptance of a new species, *H. paragallinarum* (7), for organisms requiring only the V-factor. It has been reported that the methods used in the early papers to determine X- and V-factor requirements of the causative agent of IC are defective and that *H. paragallinarum* probably never existed (24).

V-factor independent isolates of *Av. paragallinarum* have been recovered from chickens with coryza in South Africa and Mexico (35, 62, 71, 94).

The use of DNA sequencing of the 16S ribosomal RNA gene has clearly shown that there is a unique group within the bacterial family Pasteurellaceae (including both *H. paragallinarum* and *P. gallinarum*) that are associated with avian hosts—with these bacteria being rarely if ever isolated from any other host (102). Building on this knowledge, a comprehensive study based on both phenotypic and genotypic methods has clearly shown that both *H. paragallinarum* and *P. gallinarum,* as well as two other avian-associated species *P. avium* and *P. volantium,* form a new genus termed *Avibacterium* within the family Pasteurellaceae (13). The members of the new genus are *Av. paragallinarum, Av. gallinarum, Av. avium* and *Av. volantium* (13). For the rest of this text, the new terminology—*Av. paragallinarum, Av. gallinarum* and so on—will be used even if the relevant original literature has used the older terminology. There is no convincing evidence, to date, of a role in disease conditions for *Av. avium, Av. volantium* or the taxon currently known as *Avibacterium* species A (13).

Morphology and Staining

Av. paragallinarum and *Av. gallinarum* are both gram-negative nonmotile bacteria. In 24-hr cultures, both appear as short rods or coccobacilli 1–3 μm in length and 0.4–0.8 μm in width, with a tendency for filament formation. A capsule may be demonstrated in virulent strains of *Av. paragallinarum* (65, 127). *Av. paragallinarum* undergoes degeneration within 48–60 hr, showing fragments and ill-defined forms. Subcultures to fresh medium at this stage will again yield the typical rod-shaped morphology.

Growth Requirements

The reduced form of NAD (NADH; 1.56–25 μg/mL medium) (104, 116) or its oxidized form (20–100 μg/mL) (121) is necessary for the *in vitro* growth of most isolates of *Av. paragallinarum.* The exceptions are the isolates described in South Africa and Mexico that are NAD independent (35, 62, 71, 94). Sodium chloride (NaCl) (1.0–1.5%) (116) is essential for growth of *Av. paragallinarum*. Chicken serum (1%) is required by some strains (65), whereas others merely show improved growth with this supplement (21). Brain heart infusion, tryptose agar, and chicken-meat infusion are some basal media to which supplements are added (65, 84, 121). A medium that is particularly developed for the isolation of *Av. paragallinarum* in the face of contaminating gram-positive organisms has been described (146). Complex media are often used to obtain dense growth of organisms for characterization studies (10, 110, 112). The pH of various media varies from 6.9 to 7.6. A number of bacterial species excrete V-factor that will support growth of *Av. paragallinarum* on media that lack V-factor (104).

In contrast, *Av. gallinarum* has no need for NAD for in-vitro growth and grows on a range of basic media such as dextrose starch agar (64) and blood agar (98).

The determination of the growth factor requirements of the avian hemophili is not an easy process. Commercial growth factor disks used for this purpose may yield a high percentage of cultures that falsely appear to be both X- and V-factor dependent (17). The brand of disks and the medium to be used should be carefully checked for their suitability. For well-equipped laboratories, the porphyrin test (80) is recommended for X-factor testing. For classical X- and V-factor testing, the use of purified hemin and NAD as supplements to otherwise complete media may also be considered.

Av. paragallinarum is commonly grown in an atmosphere of 5% carbon dioxide; however, carbon dioxide is not an essential requirement, since the organism is able to grow under reduced oxygen tension or anaerobically (57, 104). *Av. gallinarum* also does not require carbon dioxide but a more uniform colony development does occur if isolates are incubated under an atmosphere of 5–10% carbon dioxide (13).

For *Av. paragallinarum*, the minimal and maximal temperatures of growth are 25 and 45°C, respectively, the optimal range being 34–42°C. Both *Av. gallinarum* and *Av. paragallinarum* are commonly grown at 37–38°C.

Colony Morphology

Av. paragallinarum typically gives tiny dewdrop, nonhemolytic colonies up to 0.3 mm in diameter on suitable media. In obliquely transmitted light, mucoid (smooth) colonies are iridescent, while rough colonies are noniridescent (67, 112, 122). A range of other intermediate colony forms have been observed.

Av. gallinarum colonies on serum or dextrose-starch agar are iridescent, circular, smooth, and entire and may reach up to 1.5 mm after 24 hr incubation (particularly if in 5–10% carbon dioxide) (40). A grayish-yellow pigment is typically produced (13).

Biochemical Properties

The ability to reduce nitrate to nitrite and ferment glucose without the formation of gas is common to all members of the genus *Avibacterium*. Oxidase activity and a failure to produce indole or hydrolyse urea or gelatin are also uniform characteristics (13). Considerable confusion surrounds the carbohydrate fermentation patterns of the V-factor dependent species. Much of the variability recorded in the literature may be due to the use of different basal media. False-negative results are mainly associated with poor growth and can also be a significant problem (10). In general, recent studies have used a medium consisting of a phenol red broth containing 1% (w/v) NaCl, 25 μg/mL NADH, 1% (v/v) chicken serum and 1% (w/v) carbohydrate. For routine identification, the use of the phenol red broth just described and a dense inoculum is a most suitable approach for determining carbohydrate fermentation patterns. Alternatively, agar-based methods (10, 146) may be used.

Table 20.1. Differential tests for the genus *Avibacterium*.

Taxon	*Avibacterium gallinarum*	*Avibacterium paragallinarum*	*Avibacterium volantium*	*Avibacterium avium*	*Avibacterium sp. A.*
Catalase	+	–	+	+	+
Symbiotic growth	–	V	+	+	+
ONPG	d	–	+	–	V
Acid from					
L-arabinose	–	–	–	–	+
D-galactose	+	–	+	+	+
Maltose	+	+	+	–	V
D-mannitol	–	+	+	–	V
D-sorbitol	–	+	V	–	–
Trehalose	+	–	+	+	+
α-glucosidase	+	–	+	+	+

All species are gram-negative and nonmotile. All species reduce nitrate, are oxidase positive and ferment glucose. Most isolates of *Avibacterium paragallinarum* require an enriched carbon dioxide (5–10%) atmosphere, and most will show an improved growth in the presence of 5–10% chicken serum. Most isolates of *Avibacterium gallinarum* show an improved growth in an enriched carbon dioxide (5–10%) atmosphere.

Table 20.1 presents those properties that allow a full identification of all members of the genus *Avibacterium*. The failure of *Av. paragallinarum* to ferment either galactose or trehalose and its lack of catalase clearly separate this organism from the other members of the genus.

Susceptibility to Chemical and Physical Agents

Av. paragallinarum is a delicate organism that is inactivated rather rapidly outside the host. Infectious exudate suspended in tap water is inactivated in 4 hr at ambient temperature; when suspended in saline, the exudate is infectious for at least 24 hr at 22°C. Exudate or tissue remains infectious when held at 37°C for 24 hr and, on occasion, up to 48 hr; at 4°C, exudate remains infectious for several days. At temperatures of 45–55°C, hemophili are killed within 2–10 min. Infectious embryonic fluids treated with 0.25% formalin are inactivated within 24 hr at 6°C, but the organism survives for several days under similar conditions when treated with thimerosal, 1:10,000 (157).

Av. paragallinarum may be maintained on blood agar plates by weekly passages. Young cultures maintained in a "candle jar" will remain viable for 2 wk at 4°C. Chicken embryos 6–7 days old may be inoculated with single colonies or broth cultures via the yolk sac; yolk from embryos dead in 12–48 hr will contain a large number of organisms which may be frozen and stored at –20 to –70°C or lyophilized (156). A medium that has proven to be a good suspension medium for lyophilization of *Av. paragallinarum* from agar cultures is used at the Animal Research Institute and contains 6% sodium glutamate and 6% bacteriological peptone (filter sterilized). After any storage, whether frozen or lyophilized, revival should include inoculation of a suitable liquid growth medium (egg inoculation is even better) as well as an agar medium.

There is little specific knowledge on the susceptibility of *Av. gallinarum* to either chemical or physical agents. Cultures have been shown to survive well when held at room temperature on Dorset egg slopes at room temperature (98).

Strain Classification

Antigenicity

Page (104, 105) classified his organisms of *Av. paragallinarum* with the plate agglutination test using whole cells and chicken antisera into serovars A, B, and C. While Page's serovar A strain 0083 and B strain 0222 are available today, all the serovar C strains were lost during the mid-1960s. Matsumoto and Yamamoto (88) isolated strain Modesto which was later classified as a strain of serovar C by Rimler *et al.* (114). It is now recommended to use a hemagglutination inhibition (HI) test to serotype isolates by the Page scheme (15). This HI test uses fixed chicken erythrocytes and results in fewer nontypable isolates than the original agglutination technology (15).

The distribution of Page serovars differs from country to country. Page serovar A has been reported in Malaysia (161), serovar C in Taiwan (86), serovars A and B in China (44, 164) and Germany (66), serovars A and C in Australia (14) and India (150) and serovars A, B and C in Argentina (146), Brazil (23), Ecuador (78, 136), Egypt (3) Indonesia (108, 142), Mexico (58), the Philippines (99), South Africa (36), Spain (106) and the USA (104, 105).

Another method of assigning isolates of *Av. paragallinarum* to a Page serovar is based on the use of a panel of monoclonal antibodies developed by workers in Japan (26) but the technique is available only in a few laboratories due to the limited availability of the monoclonal antibodies. Other sets of MAbs have been described but either lack serovar-specificity (38, 163) or detect only Page serovar A (140).

The suggestion that Page serovar B of *Av. paragallinarum* is not a true serovar, but rather consists of variants of serovar A or C that have lost their type-specific antigen (84, 127), is erroneous—Page serovar B is a clearly a true serovar (151).

An alternative serologic classification of *Av. paragallinarum* based on an HI test using potassium thiocyanate-treated and -sonicated cells, rabbit hyperimmune sera, and glutaraldehyde-fixed chicken erythrocytes has been developed (82). The modified Kume scheme consists of serogroups A, B, and C which match the Page serovars of A, B, and C (16). The nine currently recognized Kume serovars are A-1, A-2, A-3, A-4, B-1, C-1, C-2, C-3, and C-4 (16). Many isolates that were nontypable in the Page scheme by agglutination tests were easily typed using the Kume scheme (56).

The Kume scheme has not been widely applied, as it is technically demanding to perform. Hence, only a few laboratories are able to perform the serotyping on a routine basis. Kume serovars A-4, C-2, and C-4 have been reported in Australia (16, 56), serovars A-3, B-1 and C-1 in Ecuador (136), serovars A-1, A-2, B-1, and C2 in Germany (56, 82), serovars A-1 and C-1 in Japan (82), serovars A-1, A-2, B-1 and C-2 in Mexico (62, 135), serovars A-1, B-1, C-2, and C-3 in South Africa (56, 82), serovars A-1, B-1, and C-2 in the USA (56, 82), and serovar C-3 in Zimbabwe (29).

Other serological tests described in the literature include an agar-gel precipitin (AGP) test (68) and a serum bactericidal test (124). Neither of these tests has been widely used.

Two serotyping schemes for *Av. gallinarum* have been reported (96, 98) but there appears to have been no adoption of either of these serotyping schemes.

Immunogenicity or Protective Characteristics

Infectious coryza is relatively unique among common bacterial infections in that a bacterin (inactivated whole cell vaccine) is protective against the disease when the bacterin is adequately prepared. From the early days of bacterin production, it was obvious that protection was limited (88). Later studies confirmed a correlation between Page serovars and immunovar specificity (22, 84, 114). Chickens vaccinated with a bacterin prepared from one Page serovar were protected only against homologous challenge from that Page serovar. There is evidence that the cross-protection within Page serovar B is only partial (152).

A complete cross-protection study using the reference strains of the nine Kume serovars of *Av. paragallinarum* has been recently completed (138). Within Kume serogroup A, serovars A-1, A-2, and A-3 are strongly cross-protective while there is good cross-protection between serovars A-1 and A-4. Within Kume serogroup C, there was a good level of cross-protection for serovars C-1, C-2, and C-3, with some exceptions. Kume serovars C-1, C-2 and C-3 all provided protection against a C-1 challenge. In contrast, with the serovar C-2 and C-3 challenge, the respective homologous group was significantly better protected than the heterologous groups. The only instance of a vaccine being able to provide cross-protection across Kume serogroups that was at the same level as the homologous level was for the serovar C-4 vaccine and the serovar B-1 challenge. This study thus broadly confirmed the widely accepted dogma that serogroups A, B, and C represent three distinct immunovars.

There is only one serovar, B-1, within serogroup B of the Kume scheme. However, there are reports of undefined heterogeneity within the B serogroup. Bivalent vaccines containing Page serovars A and C provide protection against Page serovar B strain Spross, but not against two South African isolates of Page serovar B (152). Furthermore, there is only partial cross-protection within various strains of Page serovar B (152). Poor vaccine protection against IC due to serovar B strains in Argentina has been suggested to be due to antigenic differences between field isolates and the "standard" serovar B strains in commercial vaccines from North America or Europe (147). These difficulties have resulted in at least one commercial vaccine that contains multiple Page serovar B strains to provide better protection (78). Vaccination/challenge exposure studies are needed to study the antigenicity and immunospecificity of recent serovar B isolates.

In both Argentina and Brazil, there are isolates of Page serovar A that are not recognized by a monoclonal antibody specific for this serovar (23, 146), leading to speculation these "variant" Page serovar A isolates may be sufficiently different from typical serovar A vaccine strains to cause vaccine failure (146).

There have been suggestions that Kume serovar C-3 as well as other serovars of NAD-independent *Av. paragallinarum* are so antigenically different that they are causing vaccine failure (32, 36, 37, 72). However, it has been shown that a commercial vaccine, specified as containing serovars A, B and C without details of the actual strains, provided acceptable levels of protection against NAD-independent isolates of Page serovar A and Kume serovar C-3 (79).

Overall, these recent results and field observations clearly indicate the need for further vaccination/challenge studies on *Av. paragallinarum*, particularly with recent field isolates. There is no doubt that, on an on-going basis, there will continue to be debate on the topic of whether commercially available trivalent infectious coryza vaccines, containing serovars A, B and C, provide adequate protection if there are significant antigenic differences between vaccine and field strains.

There is little knowledge about the immunogenicity of *Av. gallinarum*. Autogenous vaccines have been used (98) although there is no literature on the effectiveness of these products or the existence of different immunovars.

Molecular Techniques

DNA fingerprinting by restriction endonuclease analysis has been shown to be a suitable typing technique, providing useful insights in epidemiologic studies of both *Av. paragallinarum* (20) and *Av. gallinarum* (8). Ribotyping is another molecular technique that has proven useful—providing insight into the links between the NAD-independent *Av. paragallinarum* isolates from South Africa (92), the epidemiologic relationships among Chinese isolates of *Av. paragallinarum* (90) and the heterogeneity and epidemiological links amongst *Av. gallinarum* isolates (8, 45). ERIC-PCR, a DNA fingerprinting method that uses the polymerase chain reaction technique, has been shown to be capable of typing *Av. paragallinarum* isolates (139).

These nucleic acid techniques (including the species-specific PCR discussed below) are advancing to the stage where they offer a rapid and convenient method for identification and typing. These techniques are likely to replace time-consuming and

cumbersome cultural, biochemical and serological means of identification and typing in the near future.

Pathogenicity

As a general observation, the pathogenicity of *Av. paragallinarum* can vary according to factors such as the growth conditions, passage history of the isolate and the state of the host. There is now considerable specific evidence of variation in pathogenicity amongst *Av. paragallinarum* isolates. The reference strains of Kume serovars A-1, A-4, C-1, C-2, and C-3 showed higher virulence than the strains for serovars A-2, A-3, B-1, and C-4 (137). On the basis of field observations in South Africa, Horner *et al.* (72) have suggested that the NAD-independent isolates may cause airsacculitis more commonly than the classic NAD-dependent *Av. paragallinarum* isolates. In contrast, experimental infections have shown that South African NAD-dependent isolates are more virulent than NAD-independent isolates (30, 31). The virulence of the NAD-dependent serovar C-3 strains was sufficient to cause clinical signs in vaccinated chickens and has been suggested as explaining the large number of coryza outbreaks in vaccinated flocks in South Africa (34). Experimental transformation of a serovar C-3 NAD-dependent isolate to NAD-independence markedly reduced the virulence of the transformant (143). Within a serovar, variation in virulence has also been reported—Yamaguchi *et al.* (151) found that one of four strains of *Av. paragallinarum* serovar B failed to produce clinical signs.

Experimental infections of chickens with *Av. gallinarum* generally result in little mortality (64). Field isolates from Israel have caused swollen wattles in 6-week-old chickens (98) while both an American field isolate and the type strain have caused endocarditis in mature leghorn chickens given high intravenous doses (149). Intra-muscular injection of chickens with an Argentinean isolate resulted in severe myostitis at the inoculation site (145). An American field isolate, when give by the intra-muscular route, caused severe myostitis at the inoculation site as well as pericarditis, perihepatitis, airsacculitis, and synovitis (55, 133). As the type strain failed to give similar results, there is evidence of strain variation in pathogenicity (55, 133).

Virulence Factors

A range of factors has been associated with the pathogenicity of *Av. paragallinarum.* Considerable attention has been paid to HA antigens. In both Page serovar A and C, mutants lacking HA activity have been used to demonstrate that the HA antigen plays a key role in colonization (122, 155). A gene, termed *hagA*, encoding a hemagglutinin has been identified and fully sequenced (69). The deduced sequence of this gene is closely related to the *H. influenzae* P5 protein, a protein that acts as an adhesin binding to respiratory mucin. It is possible that a similar mechanism may be involved in *Av. paragallinarum* infection (69).

The capsule has also been associated with colonization, and has been suggested to be the key factor in the lesions associated with IC (122, 129). The capsule of *Av. paragallinarum* has been shown to protect the organism against the bactericidal activity of normal chicken serum (125). It has been suggested that a toxin released from capsular organisms during *in vivo* multiplication was responsible for the clinical disease (81). The complete *Av. paragallinarum* capsule transport gene locus has been sequenced and shows high homology with other known capsule transport systems (50).

Av. paragallinarum is capable of acquiring iron from chicken and turkey transferrin, suggesting that iron sequestration may not be an adequate host defense mechanism (101). In contrast, two strains of *Av. avium* were unable to acquire iron from these transferrins despite apparently having the same receptor proteins (101).

Crude polysaccharide extracted from *Av. paragallinarum* is toxic to chickens and may be responsible for the toxic signs that may follow administration of bacterin (74). The role, if any, of this component in the natural occurrence of the disease is unknown.

A range of other putative virulence factors have been reported. A putative RTX-like protein and metalloproteases have been identified by phenotypic methods (118) while a hemocin has been confirmed by both phenotypic and genotypic methods (144). The detection of a putative RTX protein is interesting as other members of the family Pasteurellaceae have RTX proteins as major virulence factors. The hemocin of *Av. paragallinarum* is active against *Gallibacterium anatis* biovar *haemolytica* and some isolates of *P. multocida* but not against isolates of *Av. gallinarum* (144). It is possible that hemocin resistance may partly explain the reports of co-infections between *Av. paragallinarum* and *Av. gallinarum* (119, 134). The demonstration that cultures of *Av. paragallinarum* produce *in vitro* membrane vesicles that contain proteases, the putative RTX protein and hemagglutinins raises the possibility of these vesicles being involved in the development of infectious coryza (109).

There is no specific knowledge on any virulence factors associated with *Av. gallinarum*.

Pathobiology and Epidemiology

Incidence and Distribution

Infectious coryza occurs wherever chickens are raised. The disease is a common problem in the intensive chicken industry—significant problems have been reported in California, south-eastern United States and most recently in the north-eastern regions of the United States. The disease has also been reported in other, less intensive situations. As an example, infectious coryza has been a problem in kampung (village) chickens in Indonesia (108).

While reports are scarce and scattered, *Av. gallinarum* is probably present wherever chickens are raised. Outbreaks of disease associated with *Av. gallinarum* have been reported in chickens in Europe (98), North and South America (54, 145), and Africa (93).

Natural and Experimental Hosts

The chicken is the natural host for *Av. paragallinarum.* There are several reports that the village chickens of Asia are as susceptible to infectious coryza as normal commercial breeds (108, 162). While there have been reports of IC due to *Av. paragallinarum* in a number of bird species other than chickens, reviewed by

Yamamoto (158), these reports need to be interpreted carefully. As a range of hemophilic organisms, none of which are *Av. paragallinarum,* have been described in birds other than chickens (51, 63, 107), only those studies that involve detailed bacteriology can be regarded as definitive proof of the presence of *Av. paragallinarum* in birds other than chickens. The following species are refractory to experimental infection: turkey, pigeon, sparrow, duck, crow, rabbit, guinea pig, and mouse (156, 157).

Av. gallinarum has been consistently associated with chickens (45). Outbreaks of disease associated with this species have also been reported in guinea fowl in Africa (93) and turkeys in Europe (8). A single isolate has been reported from a healthy duck (97) and a goose of unspecified health status (98).

Age of Host Most Commonly Affected

All ages of chickens are susceptible to *Av. paragallinarum* (158), but the disease is usually less severe in juvenile birds. The incubation period is shortened, and the course of the disease tends to be longer in mature birds, especially hens with active egg production.

Transmission, Carriers, Vectors

Chronic or healthy carrier birds have long been recognized as the main reservoir of IC infection. The application of molecular fingerprinting techniques has confirmed the role of carrier birds in the spread of IC (20). Infectious coryza seems to occur most frequently in fall and winter, although such seasonal patterns may be coincidental to management practices (e.g., introduction of susceptible replacement pullets onto farms where IC is present). On farms where multiple-age groups are brooded and raised, spread of the disease to successive age groups usually occurs within 1–6 wk after such birds are moved from the brooder house to growing cages near older groups of infected birds (46). Infectious coryza is not an egg-transmitted disease.

Whereas the sparrow could not be implicated as a vector, epidemiologic studies suggested that *Av. paragallinarum* may be introduced onto isolated ranches by the airborne route (159).

There is no knowledge on the routes of transmission, carrier status or vectors for *Av. gallinarum*.

Incubation Period

The characteristic feature of IC is a coryza of short incubation that develops within 24–48 hr after inoculation of chickens with either culture or exudate. The latter will more consistently induce disease (112). Susceptible birds exposed by contact to infected cases may show signs of the disease within 24–72 hr. In the absence of a concurrent infection, IC usually runs its course within 2–3 wk.

Clinical Signs

The most prominent features of IC are an acute inflammation of the upper respiratory tract including involvement of nasal passage and sinuses with a serous to mucoid nasal discharge, facial edema, and conjunctivitis. Figure 20.1 illustrates the typical facial edema. Swollen wattles may be evident, particularly in males. Rales may be heard in birds with infection of the lower respiratory tract.

A swollen head-like syndrome associated with *Av. paragallinarum* has been reported in broilers in the absence of avian pneumovirus, but in the presence or absence of other bacterial pathogens such as *M. synoviae* and *M. gallisepticum* (52, 119). Arthritis and septicemia have been reported in broiler and layer flocks, respectively, in which the presence of other pathogens has contributed to the disease complex (119).

Birds may have diarrhea, and feed and water consumption usually is decreased; in growing birds this means an increased number of culls, and in laying flocks a reduction in egg production (10 to 40%). A foul odor may be detected in flocks in which the disease has become chronic and complicated with other bacteria.

The most common signs seen in outbreaks of disease where *Av. gallinarum* has played a potential role have been those of an acute respiratory disease, coughing and sneezing, with some outbreaks involving peri-orbital swelling and keratoconjunctivitis. Swollen wattles have been reported in cases in Israel (98) and Africa (93).

Morbidity and Mortality

IC is usually characterized by low mortality and high morbidity. Variations in age and breed may influence the clinical picture (9). Complicating factors such as poor housing, parasitism, and inadequate nutrition may add to severity and duration of the disease. When complicated with other diseases such as fowlpox, infectious bronchitis, laryngotracheitis, *Mycoplasma gallisepticum* infection, and pasteurellosis, IC is usually more severe and prolonged, with resulting increased mortality (119, 156). Even in the absence of any other pathogen, older birds can suffer a high mortality as shown by an outbreak in California where the total mortality reached 48% (27).

While not common, high mortalities have been seen associated with *Av. gallinarum* in broilers (up to 34%) (54) and in turkeys (up to 26%) (8).

Pathology

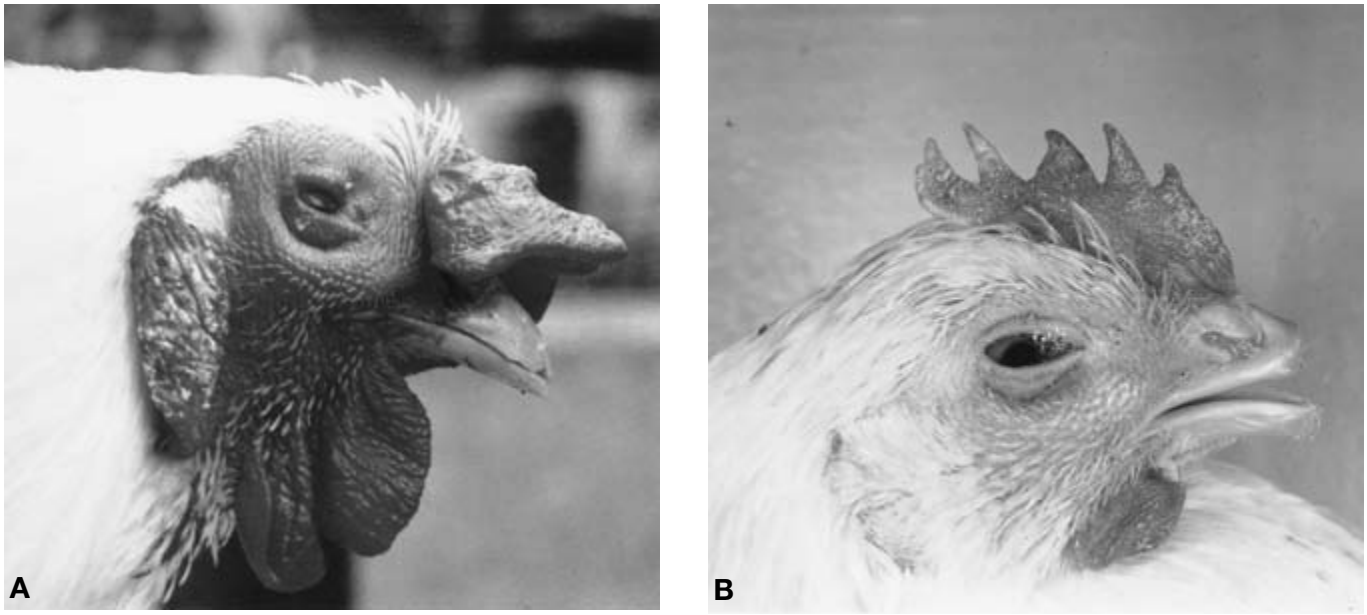
Gross

Av. paragallinarum produces an acute catarrhal inflammation of mucous membranes of the nasal passages and sinuses. There is frequently a catarrhal conjunctivitis and subcutaneous edema of the face and wattles. Typically, pneumonia and airsacculitis are rarely present; however, reports of outbreaks in broilers have indicated significant levels of condemnations (up to 69.8%) due to airsacculitis (Figure 20.2), even in the absence of any other recognized viral or bacterial pathogens (52, 70).

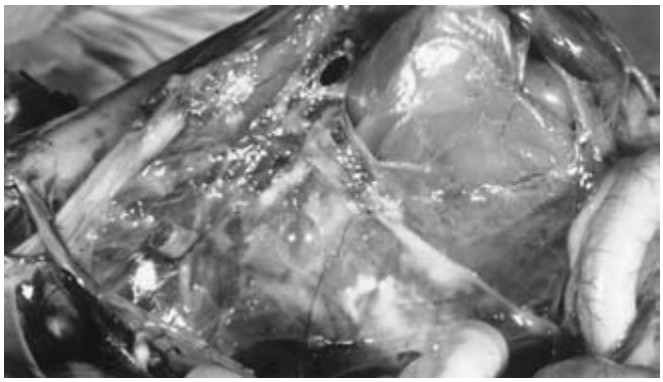
The lesions associated with *Av. gallinarum* infections have been diverse and include airsacculitis, conjunctivitis, pericarditis, perihepatitis and sinusitis (45).

Microscopic

Fujiwara and Konno (60) studied the histopathologic response of chickens from 12 hr to 3 mo after intranasal inoculation with *Av. paragallinarum*. Essential changes in the nasal cavity, infraorbital sinuses, and trachea consisted of sloughing, disintegration, and hyperplasia of mucosal and glandular epithelia, and edema and hyperemia with heterophil infiltration in the tunica propria of



20.1. Chickens artificially infected with *Avibacterium paragallinarum*. A. Mature male with coryza and facial edema. B. Mature female showing conjunctivitis, nasal discharge, and open-mouth breathing.



20.2. Field infection with IC showing caseopurulent air sac lesions.

the mucous membranes. Pathologic changes first observed at 20 hr reached maximum severity by 7–10 days, with subsequent repair occurring within 14–21 days. In birds with involvement of the lower respiratory tract, acute catarrhal bronchopneumonia was observed, with heterophils and cell debris filling the lumen of secondary and tertiary bronchi; epithelial cells of air capillaries were swollen and showed hyperplasia. Catarrhal inflammation of air sacs was characterized by swelling and hyperplasia of the cells, with abundant heterophil infiltration. In addition, a pronounced infiltration of mast cells was observed in the lamina propria of the mucous membrane of the nasal cavity (130). The products of mast cells, heterophils, and macrophages may be responsible for the severe vascular changes and cell damage leading to coryza. A dissecting fibrinopurulent cellulitis similar to that seen in chronic fowl cholera has been reported in broiler and layer chickens (52).

Shivaprasad and Droual (133) examined the histopathology caused by experimental infection with the *Av. gallinarum* strain associated with severe mortality in broilers in California. The lesions caused by this field isolate were more severe than those associated with the type strain and included severe subacute to chronic pyogranulomatous pneumonia, airsacculitis, pericarditis, perihepatitis, synovitis, and myositis. There was severe lymphoid depeletion of the bursa of Fabricius (133).

Immunity

Chickens that have recovered from active infection with *Av. paragallinarum* possess varying degrees of immunity to reexposure. Pullets that have experienced IC during their growing period are generally protected against a later drop in egg production. Resistance to reexposure among individual birds may develop as early as 2 wk after initial exposure by the intrasinus route (120).

It has been shown that experimentally infected chickens develop a cross-serovar (Page scheme) immunity (113). In contrast, as discussed earlier, bacterins provide only serovar-specific immunity (114). This suggests that cross-protective antigens are expressed *in vivo* that are either not expressed or expressed at very low levels *in vitro*.

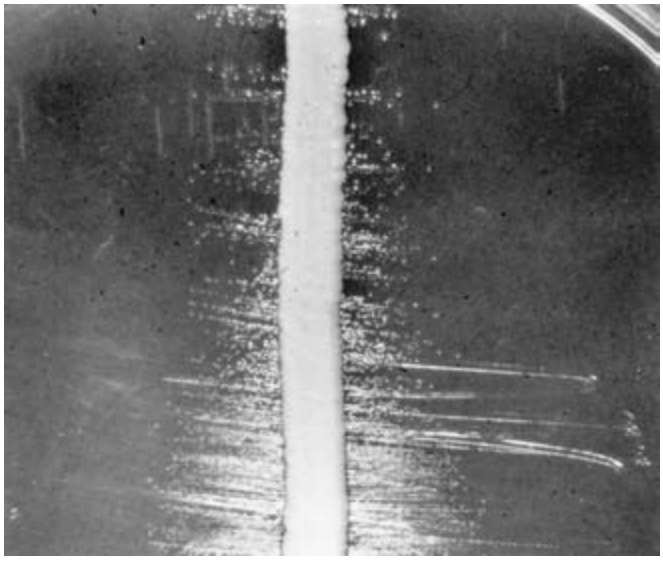
The protective antigens of *Av. paragallinarum* have not been definitively identified. It has been suggested that the capsule of *Av. paragallinarum* contains protective antigens (123). Using both a Page serovar A and C strains, a crude polysaccharide extract was shown to provide serovar-specific protection (74).

Considerable attention has been paid to the role of HA antigens as protective antigens. It has been long noted that for Page

serovar A organisms, there is a close correlation between HI titer and both protection (85) and nasal clearance of the challenge organism (81) in vaccinated chickens. Purified HA antigen from a Page serovar A organism has been shown to be protective (75). Takagi and colleagues have shown that a monoclonal antibody specific for the HA of Page serovar A provides passive protection, and that the HA antigen purified by use of this antibody is also protective (140, 141). It is worth noting that vaccines that do not stimulate HI titers can still be protective (61, 138) suggesting that other antibodies do have a role in protection.

Based on studies conducted to date, there is considerable evidence that the protective antigens of *Av. paragallinarum* are surface located. The antigens implicated have been the antigens detected during Page serotyping, HA antigens, and some component or components of the polysaccharide content of the cell. It seems probable that a number of different antigens (outer-membrane proteins, polysaccharides, lipopolysaccharides) are all likely to be involved.

While autogenous bacterins of *Av. gallinarum* have been used and have been shown to stimulate antibodies (98), there is no detailed understanding of the efficacy of these products or the key antigens for protection.



20.3. Satellite phenomenon. Tiny dewdrop colonies of *Avibacterium paragallinarum* growing adjacent to *Staphylococcus* culture (broad streak) on a blood agar plate.

Diagnosis

Isolation and Identification of Causative Agent

While *Av. paragallinarum* is considered to be a fastidious organism, it is not difficult to isolate, requiring simple media and procedures. Specimens should be taken from two or three chickens in the acute stage of the disease (1–7 days' incubation). The skin under the eyes is seared with a hot iron spatula and an incision made into the sinus cavity with sterile scissors. A sterile cotton swab is inserted deep into the sinus cavity where the organism is most often found in pure form. Tracheal and air sac exudates also may be taken on sterile swabs. Where transport delays are possible, the swabs should be placed in a commercial transport medium containing supplements to improve viability of the organism (39). The swab is streaked on a blood agar plate, which is then cross-streaked with a *Staphylococcus* culture and incubated at 37°C in a large screw-cap jar in which a candle is allowed to burn out (Figure 20.3). *Staphylococcus epidermidis* (104) or *S. hyicus* (21), which are commonly used as "feeders," should be pretested because not all strains actively produce the V-factor. Terzolo *et al.* (146) have reported the successful use of an isolation medium that contains selective agents that inhibit the growth of gram-positive bacteria. This medium has the advantage of not using either a "feeder" organism or additives such as NADH.

At the simplest level, IC may be diagnosed on the basis of a history of a rapidly spreading disease in which coryza is the main manifestation, combined with the isolation of a catalase-negative bacterium showing satellitic growth. At this level, the sinus exudate or culture should be inoculated into two or three normal chickens by the intrasinus route. The production of a coryza in 24–48 hr is diagnostic; however, the incubation period may be delayed up to 1 wk if only a few organisms are present in the inoculum, such as in long-standing cases.

Better equipped laboratories should attempt a more complete biochemical identification as described earlier. Additional studies of this nature are essential when isolates of NAD-independent *Av. paragallinarum* are suspected. To perform this biochemical testing, the suspect isolates are best grown in pure culture on medium that does not require the addition of a nurse colony. While many different media have been developed to support the growth of *Av. paragallinarum*, the medium called TM/SN (21) has proven very effective. The medium described by Terzolo *et al.* (146) is particularly suited for those laboratories where the cost of such ingredients as NADH and bovine serum albumin is too expensive. The carbohydrate fermentation tests shown in Table 20.1 can be done in either a phenol red broth base (112) or in an agar plate format (10). The agar plate method can be performed in conventional petri dishes (9 cm), allowing multiple isolates to be tested at once, or in small petri dishes (2 cm), allowing one to three isolates to be economically characterized. The agar plate method (10) has also been modified to be performed as a tube method (146).

A PCR test that is specific for *Av. paragallinarum*, rapid and able to detect all known variants is available (42). The PCR, termed the HP-2 PCR, has been validated for use on colonies on agar or on mucus obtained from squeezing the sinus of live birds (42). When used directly on sinus swabs obtained from artificially-infected chickens in pen trials performed in Australia, the HP-2 PCR has been shown to be the equivalent of culture—but much more rapid (42). When used in China, direct PCR examination of sinus swabs outperformed traditional culture when used on routine diagnostic submissions (41). The problems of poor samples, delayed transport and poor quality (but expensive) media mean that culture will have a higher failure rate in developing countries than in developed countries—making the PCR an attractive diagnostic option.

The HP-2 PCR is a robust test—sinus swabs stored for up to 180 days at 4°C or –20°C were positive in the PCR (43). In contrast, culture of known positive swabs failed to detect *Av. paragallinarum* after 3 days of storage at 4°C or –20°C (43).

The HP-2 PCR has proven very useful in South Africa where the diagnosis of infectious coryza is complicated by the presence of NAD-independent *Av. paragallinarum* and *Ornithobacterium rhinotracheale* as well as the traditional form of NAD-dependent *Av. paragallinarum* (91).

Isolation of *Av. gallinarum* is best done using sheep blood agar plates incubated at 37°C under a 5–10% carbon dioxide atmosphere. Phenotypic tests (see Table 20.1) should be performed using conventional methodologies. No molecular diagnostic test has been developed for *Av. gallinarum*.

Serology

There is no totally suitable serological test for the diagnosis of infectious coryza. However, despite this absence of a "perfect" test, serological results are often useful for retrospective/epidemiological studies in the local area. A review of the techniques that have been used in the past is presented by Blackall *et al.* (19).

At this time, the best available test methodology is the HI test. While a range of HI tests have been described, three main forms of HI tests have been recognized—these being termed simple, extracted and treated HI tests (25). Full details of how to perform these tests are available elsewhere (25). In the following text, the advantages and disadvantages of the three HI tests are briefly and critically discussed.

The simple HI is based on whole bacterial cells of Page serovar A *Av. paragallinarum* and fresh chicken erythrocytes (77). Although simple to perform, this HI test can only detect antibodies to serovar A. The test has been widely used to both detect infected as well as vaccinated chickens (19). A variation of this test (whole bacterial cells and glutaraldehyde-fixed chicken erythrocytes) has been shown to detect antibodies due to all nine Kume serovars in vaccinated chickens (138).

The extracted HI test is based on KSCN-extracted and sonicated cells of *Av. paragallinarum* and glutaraldehyde-fixed chicken erythrocytes (128). This extracted HI test has mainly been validated for the detection of antibodies to Page serovar C organisms. The test has been shown to be capable of detecting a serovar-specific antibody response in Page serovar C vaccinated chickens (128). A major weakness with this assay is that, in chickens infected with serovar C, the majority of the birds remain seronegative (153).

The treated HI test is based on hyaluronidase-treated whole bacterial cells of *Av. paragallinarum* and formaldehyde-fixed chicken erythrocytes (154). The extracted HI has not been widely used or evaluated. It has been used to detect antibodies to Page serovars A, B, and C in vaccinated chickens with only serovar A and C vaccinated chickens yielding high titers (152). The test has been used to screen chicken sera in Indonesia for antibodies arising from infection with serovars A and C (142).

Vaccinated chickens with titers of 1:5 or greater in the simple or extracted HI tests have been found to be protected against subsequent challenge (128). There is not enough knowledge or experience yet to draw any sound conclusions on whether there is a correlation between titer and protection for the treated HI test.

Overall, the serological test of choice remains either the simple HI test (77) for either infections or vaccinations associated with serovar A, the extracted or treated HI tests (128, 154) for vaccinations associated with serovar C and only the treated HI test (154) for infections associated with serovar C. There has been so little work performed on serological assays for infections or vaccinations associated with serovar B that it is not possible to recommend any test.

Both plate agglutination and gel precipitin tests have been described for the detection of antibodies to *Av. gallinarum* (98) but there has been no apparent use of these assays.

Differential Diagnosis

Infectious coryza must be differentiated from other diseases such as chronic respiratory disease, chronic fowl cholera, fowlpox, ornithobacterosis, swollen head syndrome, and A-avitaminosis, which can produce similar clinical signs. Since *Av. paragallinarum* infections often occur in mixed infections, one should consider the possibility of other bacteria or viruses as complicating IC, particularly if mortality is high and the disease takes a prolonged course (see Pathogenicity; Morbidity and Mortality).

As the conditions linked with *Av. gallinarum* are generally linked with upper respiratory disease, a similar range of disease conditions as those listed above need to be considered. A thorough bacteriological characterization is necessary to ensure correct identification of suspect *Av. gallinarum* isolates.

Intervention Strategies

Management Procedures

Since recovered carrier birds are the main source of infectious coryza, practices such as buying breeding males or started chicks from unknown sources should be discouraged. Only day-old chicks should be secured for replacement purposes unless the source is known to be free of IC. Isolation rearing and housing away from old stock are desirable practices. To eliminate the agent from a farm, it is necessary to depopulate the infected or recovered flock(s) because birds in such flocks remain reservoirs of infection. After cleaning and disinfection of the equipment and houses, the premises should be allowed to remain vacant for 2–3 wk before restocking with clean birds.

Recent studies have shown that the continuous use of appropriate disinfectants in drinking water and by daily fogging can reduce the duration and severity of clinical signs of infectious coryza (33, 73).

Vaccination

Types of Vaccines

Commercial IC bacterins are widely available. As the literature of the various factors influencing the efficacy of bacterins has been reviewed (12), only key points are considered here. Most commercial products are currently based on broth-grown cultures. They must contain at least 10^8 colony-forming units/mL to be ef-

fective (88). The following section reviews only the literature on broth-based bacterins.

There is disagreement in the literature as to the effect of different inactivating agents on the efficacy of bacterins. Thimerosal has been shown to be effective (22, 48, 88), as has formalin (47, 117). In three of the four studies directly comparing formalin and thimerosal, formalin reduced the efficacy of the vaccines (22, 48, 87, 88). These studies suggest that while vaccines containing formalin as the inactivating agent can be protective, it is possible that a similar vaccine containing thimerosal would be even more efficient.

A number of adjuvants have been shown to be effective for IC bacterins, in particular, aluminium hydroxide gel, mineral oil, and saponin (18). The report of mineral oil being less effective than aluminium hydroxide gel (111) may have resulted from a formulation problem rather than any inherent deficiency in the ability of mineral oil to act as an effective adjuvant. As with any bacterin that contains adjuvants, particularly mineral oil, the potential adverse reaction at the site of injection (53) should be considered when using such products.

As inactivated IC bacterins provide, at best, protection only against the Page serovars included in the vaccine, it is vital that bacterins contain the Page serovars present in the target population. The confirmed existence of Page serovar B as a true serovar with full pathogenicity, as well as its widespread occurrence, means that this serovar must be included in inactivated bacterins in areas where serovar B is present. However, since different strains of serovar B provide only partial cross-protection among themselves (152), it may be necessary to prepare an autogenous bacterin for use in areas where the B serovar is endemic or consider commercial bacterins that contain multiple serovar B strains (78). The finding that some Kume serovars within serogroup C are not fully cross-protective (138) needs to be considered in areas where multiple Kume C serovars are known to exist.

Since dissociation of *Av. paragallinarum* has been reported (126), care should be taken in selecting the proper seed culture, media, and incubation period to obtain the most immunogenic product.

Mixed bacterins containing inactivated infectious bronchitis virus, Newcastle disease virus, and *Av. paragallinarum* have been described (103, 160). A combined *Av. paragallinarum–M. gallisepticum* bacterin was reported to provide protection against transient and chronic coryza (115). However, antibody response to *Av. paragallinarum* was suppressed in chickens inoculated with a similar product (89).

There appears to have been no widespread use of *Av. gallinarum* vaccines.

Field Vaccination Protocol and Regimes

IC bacterins are generally injected in birds between 10 and 20 wk of age and yield optimal results when given 3–4 wk prior to an expected natural outbreak. Two injections given approximately 4 wk apart before 20 wk of age seem to result in better performance of layers than a single injection. When administered to growing birds, the bacterin reduces losses from complicated respiratory disease. Both subcutaneous and intramuscular routes have been effective (22, 48, 88). Injection of the bacterin into the leg muscle gave better protection than when injected into the breast muscle (76). The intranasal route was not effective (22). Oral delivery of an IC bacterin was effective, but this route required 100 times as many cells as with the parenteral route (100). Significant immunity has been demonstrated for about 9 mo following vaccination (22, 83, 88).

Treatment

Various sulfonamides and antibiotics are useful in alleviating the severity and course of IC and have been reviewed (19). It should be noted drug resistance in *Av. paragallinarum* does occur (11) and hence the performance of antimicrobial sensitivity tests is recommended. Relapse often occurs after treatment is discontinued and the carrier state is not eliminated (157). Erythromycin and oxytetracycline are two commonly used antibiotics.

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Chapter 21

Mycoplasmosis

Introduction

Stanley H. Kleven

Mycoplasmas are very small prokaryotes devoid of cell walls, bounded by a plasma membrane only (24). This accounts for the "fried egg" type of colony morphology, resistance to antibiotics that affect cell wall synthesis, and complex nutritional requirements. Mycoplasmas tend to be quite host-specific; some infect only a single species of animal, while others may have the ability to infect several different host species. They are found in humans, many animal species, plants, and insects. In general, mycoplasmas colonize mucosal surfaces and most species are noninvasive. Some species, including *Mycoplasma gallisepticum* (32), are now known to have the ability to penetrate cells.

Characterization

Mycoplasma species from avian sources generally require a protein-rich medium containing 10–15% added animal serum. Further supplementation with some yeast-derived component is often beneficial. Growth of *M. synoviae* requires the addition of nicotinamide adenine dinucleotide (NAD) (see *M. synoviae* section). A medium described by Frey (13) or a medium described by Bradbury (1) is commonly used for the cultivation of avian mycoplasmas.

Mycoplasma organisms tend to grow rather slowly, usually prefer 37–38°C, and are rather resistant to thallium acetate and penicillin, which are frequently employed in media to retard growth of contaminant bacteria and fungi. Colonies form on agar media after 3–10 days at 37°C; however, nonpathogenic species such as *M. gallinarum* and *M. gallinaceum* may develop colonies within 1 day (*M. gallinarum* and *M. gallinaceum* are frequently isolated as contaminants during attempts to isolate pathogenic avian mycoplasmas). Typical colonies are small (0.1–1.0 mm), smooth, circular, and somewhat flat with a denser central elevation (see Fig. 21.1). Variations in colony morphology have been described, but cannot be relied upon to differentiate the various species. Individual cells vary from 0.2 to 0.5 µm and are basically coccoid to coccobacilliform, but slender rods, filaments, and ring forms have been described.

Fermentation of carbohydrates is variable, but all species may be divided into those that ferment glucose with acid production and those that do not. Glucose is frequently added to broth media to enhance growth of the carbohydrate-fermenting species and to provide an indication of growth when glucose fermentation produces acid in media containing added phenol red. Phosphatase activity is often present, as is arginine decarboxylase. Most species that do not ferment glucose use the amino acid arginine as their major source of energy. *M. iowae* and some other species, however, ferment glucose and hydrolyze arginine.

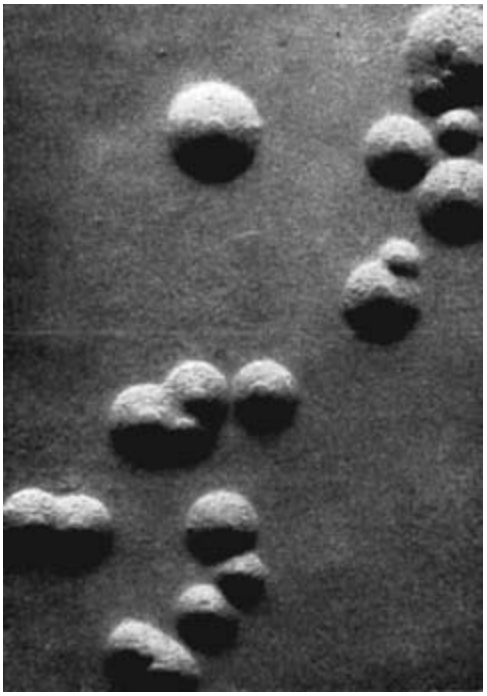
One useful characteristic of *M. gallisepticum, M. meleagridis,* and *M. synoviae* is hemagglutination of erythrocytes from chickens or turkeys. Hemagglutinating antigens are used for hemagglutination-inhibition serologic tests for these three pathogenic species.

Direct staining of mycoplasma colonies on agar surfaces or colony imprints with specific fluorescent antibody (7, 29) is most commonly used to determine the species of avian mycoplasma isolates. Other suitable methods include growth inhibition (6), immunodiffusion (20), and others. More recently, molecular methods such as sequencing of the 16S rRNA gene (14), DNA probes (15), and polymerase chain reaction (18, 19, 34) have been used.

Classification

Mycoplasmas are members of the class Mollicutes, Order I, Mycoplasmatales. Genus I, *Mycoplasma,* has more than 120 species, a DNA G+C content of 23–40%, a genome size of 600–1350 kb, requires cholesterol for growth, occurs in humans and animals, and has a usual optimum growth temperature of 37°C. Genus II, *Ureaplasma,* is differentiated on the basis of hydrolysis of urea. Acholeplasmas are classified in Order III, Acholeplasmatales, family I, Acholeplasmataceae, genus I, *Acholeplasma.* They are characterized by lack of a growth requirement for cholesterol (24). Phylogenetic analysis of the 16S ribosomal RNA gene has been used to analyze genetic relationships among mycoplasmas (30).

Earlier serotype designations for avian mycoplasmas (8) have now been replaced by species names. A current listing of avian mycoplasma species is found in Table 21.1. In addition, there are numerous mycoplasma isolates from various species of birds, including strain 1220, a pathogen of domestic geese (28), isolates



21.1. Colonies of *Mycoplasma gallisepticum* on agar. ×40 (Hofstad)

from various avian species including ratites and penguins, as well as unidentified isolates from domestic poultry.

The most up to date listing of *Mycoplasma* species can be found on the web site of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). Minimum requirements for the description of new species of *mycoplasma* are determined by the International Committee on Systematics of Prokaryotes Subcommittee on the Taxonomy of *Mollicutes* (31). An updated version of the guidelines is complete and should be found in the *International Journal of Systematic and Evolutionary Microbiology* within the next year.

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Table 21.1. Characteristics of avian mycoplasmas.

Species	Usual host	Glucose fermentation	Arginine hydrolysis	Reference
A. laidlawii[a]	Various	+	–	(27)
M. anatis	Duck	+	–	(25)
M. anseris	Goose	–	+	(4)
M. buteonis	Buteo hawk	+	–	(23)
M. cloacale	Turkey, goose	–	+	(3)
M. columbinasale	Pigeon	–	+	(16)
M. columbinum	Pigeon	–	+	(26)
M. columborale	Pigeon	+	–	(26)
M. corogypsi	Black vulture	+	–	(22)
M. falconis	Saker falcon	–	+	(23)
M. gallinarum	Chicken	–	+	(12)
M. gallinaceum	Chicken	+	–	(16)
M. gallisepticum	Chicken, turkey, house finch, other	+	–	(9)
M. gallopavonis	Turkey	+	–	(16)
M. glycophilum	Chicken	+	–	(10)
M. gypis	Griffon vulture	–	+	(23)
M. imitans	Duck, goose, partridge	+	–	(5)
M. iners	Chicken	–	+	(9)
M. iowae	Turkey	+	+	(16)
M. lipofaciens	Chicken	+	+	(2)
M. meleagridis	Turkey	–	+	(33)
M. pullorum	Chicken	+	–	(16)
M. sturni	European starling	+	–	(11)
M. synoviae	Chicken, turkey	+	–	(16, 21)
U. gallorale[b]	Chicken	–	–	(17)

[a] *Acholeplasma* species do not require sterols for growth.
[b] *Ureaplasma* species are characterized by splitting of urea.

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Mycoplasma gallisepticum Infection

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Introduction

Definition and Synonyms

Mycoplasma gallisepticum (MG) infections are commonly known as "chronic respiratory disease" (CRD) of chickens and "infectious sinusitis" of turkeys. *M. gallisepticum* diseases are characterized by respiratory rales, coughing, nasal discharge, and conjunctivitis; and frequently in turkeys, infraorbital sinusitis. Clinical manifestations are usually slow to develop and the infection or disease may have a long course. "Air sac disease" describes a severe airsacculitis that is the result of MG or *Mycoplasma synoviae* infection complicated by a respiratory virus infection (e.g. infectious bronchitis or Newcastle disease) and usually *Escherichia coli*.

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Economic Significance

M. gallisepticum is the most pathogenic and economically significant mycoplasmal pathogen of poultry. Airsacculitis in chickens or turkeys resulting from MG infections, with or without complicating pathogens, causes increased condemnations at processing. Economic losses from condemnations or downgrading of carcasses, reduced feed and egg production efficiency, and increased medication costs are additional factors that make this one of the costliest disease problems confronting commercial poultry production worldwide. Prevention and control programs, which may include surveillance (serology; culture, isolation and identification) and vaccination, account for additional costs.

Public Health Significance

M. gallisepticum infects a relatively narrow range of exclusively avian host species and has no public health significance.

History

The first accurate description of the disease in turkeys was probably made in 1905 by Dodd (98) in England, and termed "epizootic pneumoenteritis." Dickinson and Hinshaw (96) named the disease "infectious sinusitis" of turkeys in 1938. In 1935, Nelson (303) described coccobacilliform bodies associated with an infectious coryza of chickens. Later he associated the organisms with a coryza of slow onset and long duration, and eventually was able to grow the coccobacilliform bodies in embryonating eggs, tissue culture, and cell-free medium. In 1943 Delaplane and Stuart (93) cultivated in embryos an agent isolated from chickens with CRD, and later from turkeys with sinusitis. In the early 1950s Markham and Wong (268) and Van Roekel and Olesiuk (389) reported the successful cultivation of the organisms from chickens and turkeys, and suggested they were both members of the pleuropneumonia group (*Mycoplasma* spp.).

Etiology

Classification

M. gallisepticum is an avian pathogen within the genus *Mycoplasma* (class Mollicutes) which includes approximately 100 other species infecting animals (including humans), insects or plants (326). Mollicutes are eubacteria without cell walls and the smallest self-replicating (can be grown on artificial cell-free media) prokaryotes (326). *M. gallisepticum* was first classified and differentiated from other avian mycoplasmas by serotyping (408, 409) and was commonly designated serotype A (210, 410). The species designation *M. gallisepticum* was made in 1960 by Edward and Kanarek (103). In 1993 mycoplasmas with phenotypic and antigenic similarities to MG were differentiated by molecular techniques and designated *M. imitans* (48, 161).

Mycoplasma phylogeny and taxonomy continue to be reexamined by the application of molecular tools eg. DNA sequence analysis of the 16S rRNA gene (326, 393), 16S rRNA PCR and denaturing gradient gel electrophoresis (278), and tRNA gene PCR (358). The complete genome sequence has been determined for MG strain R_{low} (319), and a database dedicated to the comparative genomics of Mollicutes, including MG, has been established (32).

Morphology and Staining

M. gallisepticum stains well with Giemsa and by light microscopy organisms generally appear coccoid, approximately 0.25–0.5 μm in size. Based on electron microscopy (EM) studies Tajima *et al.* (368) described capsular material associated with MG cells in contact with chicken tracheal epithelium. Also by EM organisms appear round or may show a filamentous or flask-shaped polarity of the cell body. This polarity appears prior to division (291) and is due to the presence of well-organized terminal organelles (blebs or tip structures) (69). Such structures are involved in motility, chemotaxis, (209, 232, 287) and host-pathogen interactions eg. cytadherence (hence also termed attachment organelles) and pathogenicity (24).

Growth Requirements

M. gallisepticum replication requires a rather complex medium usually enriched with 10–15% heat-inactivated swine, horse, or avian serum. Several types of liquid or agar media will support the growth of avian mycoplasmas (217). Frey *et al.* (129) developed a culture medium that incorporated all essential ingredients including yeast autolysate and glucose. When prepared with 10–15% swine serum, it is a convenient and very effective medium for cultivation of most mycoplasmas. Extraneous bacterial and fungal contamination usually is controlled by the inclusion of thallous acetate (1:4000) and penicillin (up to 2000 IU/ml). *M. gallisepticum* is one of the avian *Mycoplasma* spp. that ferments glucose which lowers the pH causing the phenol red indicator to change from red to orange/yellow making it possible to visually detect growth in broth. Optimal growth in broth medium generally occurs at approximately pH 7.8 with aerobic incubation at 37°C and usually is evident in 3–5 days, but some field isolates may take longer and require multiple passages (245). Growth may not be evident, but 2 or 3 serial passages at 5–7-day intervals may increase the number of isolations. Direct plating of exudates or tissue swabs onto mycoplasma agar may result in colonies after 4–5 days of incubation, but initial culture in broth is generally a more sensitive isolation method. Inoculated plates should be covered and incubated at 37°C in a very moist atmosphere and may require at least 3–7 days of incubation before typical mycoplasmal colonies are sufficiently large to be observed with a dissecting microscope (217). *M. gallisepticum* may also be isolated or propagated in embryonated chicken eggs. See "Isolation and Identification of Causative Agent".

Colony Morphology

M. gallisepticum colonies form on agar medium inoculated directly or following passage from broth or agar cultures (217). It often is very difficult to obtain colony growth directly from clinical specimens. Evidence of colony growth is best studied with the aid of a dissecting microscope with indirect lighting. Characteristic colonies appear as tiny, smooth, circular, translucent masses that may have a dense central area ("fried egg" appearance). They rarely are more than 0.2–0.3 mm in diameter and fre-

quently occur in ridges along the streak line, because closely adjacent colonies readily coalesce. Variations in colony morphology of isolates representing various species of avian mycoplasmas have been noted, but *Mycoplasma* spp. designations cannot be determined by colony characteristics alone.

Biochemical Properties

Biochemical and related biologic properties of MG have been described (171, 410). *M. gallisepticum* ferments glucose and maltose, with production of acid but not gas. It does not ferment lactose, dulcitol, or salicin. Sucrose is rarely fermented; results with galactose, fructose, trehalose, and mannitol are variable. It does not hydrolyze arginine and is phosphatase-negative. It reduces 2,3,5-triphenyl tetrazolium and neotetrazolium. *M. gallisepticum* causes complete hemolysis of horse erythrocytes incorporated into agar medium and agglutinates turkey and chicken erythrocytes.

Susceptibility to Chemical and Physical Agents

It is assumed that most of the commonly employed chemical disinfectants are effective against MG. Inactivation has been produced by phenol, formalin, ß-propiolactone, and merthiolate. Resistance to penicillin and low concentration (1:4000) of thallous acetate make these valuable additives to mycoplasma culture media as inhibitors of bacterial and fungal contamination.

Broth cultures of MG remained viable for 2–4 years when stored at –30°C, and viable organisms were recovered from lyophilized broth culture stored at 4°C for at least 7 years, and from lyophilized infective chicken turbinates stored at 4°C for 13–14 years (410). Broth cultures of MG that had been frozen at –60°C since 1965 were viable upon subculturing more than 20 years later (413). Lyophilized broth cultures including MG, *M. synoviae* (MS), and *M. meleagridis* (MM) were routinely found to be viable when subcultured at 10–15 years. However, loss of MG viability in liquids should be anticipated depending on the strain, medium or diluent, and temperature. *M. gallisepticum* F strain in powdered skim milk, phosphate-buffered saline (PBS), tryptose phosphate broth, and distilled water stored at 4°, 22°, and 37°C was stable in all diluents for 24 hours when stored at 4° or 22° but not 37°C (213). An MG inoculum stock culture showed a post-thaw drop of titer (color changing units) of 10^3 in 24 hours at 4°C and 10^5 at room temperature (242).

M. gallisepticum was inactivated in infected chicken hatching eggs that reached 45.6°C during a 12–14 hour heating procedure (411).

Antigenic Structure and Toxins

The antigenic characteristics of MG and relatively species-specific polyclonal antibody response to the organism have been used to identify both the organism (growth inhibition and immunofluorescence tests) and immunologic response (serologic tests) to infection (14, 217). These tests were empirically developed and optimized to enhance their sensitivity and species-specificity with little or no knowledge of the organism's antigenic structure. Proteins constitute more than two-thirds of the mycoplasma membrane mass, with the rest being membrane lipids (328). The plasma membrane of MG contains approximately 200 polypeptides (177) that typically for mycoplasmas are associated with surface antigenic variation, adhesion to host cells, motility, and nutrient transport (287, 400).

Considerable effort has been made to identify MG antigens, especially those with adhesin or hemagglutinin properties, which may play key roles in the pathogenesis of, and immune response to, infection. Adhesins are integral membrane proteins having regions exposed on the cell surface that attach to receptor sites on host epithelial cells, which allow for colonization and infection, and as such are considered important virulence factors and antigens. *M. gallisepticum* proteins or lipoproteins with molecular weights ranging from 60–75 kDa have been described as immunodominant adhesins or hemagglutinins (21, 29, 51, 126, 208, 270).

Originally, two MG gene families, pMGA and *pvpA*, were described which encode major surface proteins with pathogenic, antigenic, and immune evasion properties (270, 273, 419). The pMGA multigene family was found to encode variant copies of a major cell surface lipoprotein hemagglutinin of 67 kDa (p67) (33, 148, 177, 272). Immunoblotting techniques revealed that the surface antigens p52 and p67 (pMGA) were specific to MG and the closely related species *M. imitans*. No antigenic difference was revealed within these species with anti-p52 serum, while anti-p67 serum confirmed the antigenic variability of p67 (178). The pMGA gene family occupies a minimum of 7.7% of the genome of strain F and 16% of the genome of strain R (33), which are significant genomic commitments to antigenic variability and hypothesized function of immune evasion (273). The pMGA gene family also provides a mechanism for rapid and reversible switches in its expression of proteins (antigenic switching) in response to antibodies or other environmental cues (146, 147, 271). In 2003 the pMGA gene and protein were renamed *vlhA* and VlhA respectively (319). The *vlhA* gene family encodes hemagglutinin in MG, and the *vlhA* genes are located in several loci around the chromosome and antigenic variation is generated by alternating transcription of over 40 translationally competent genes (7, 307).

PvpA is an MG size-variable integral membrane protein that shows high-frequency phase variation in its expression and adds to the complexity of antigenic variation in MG (241, 419). Antigenic variation and expression of PvpA and p67a, major immunogenic surface proteins, were correlated with antibody response *in vivo*, suggesting that immune modulation may have a key role in generating surface diversity (241). Size variation of the PvpA protein was observed among MG strains, ranging from 48 to 55 kDa; by immunoelectron microscopy the PvpA protein was localized on the mycoplasma cell surface at the terminal tip structure (46). The preceding information and that from many other reports indicates that the MG genome is highly committed to antigenic variation and variable expression of surface proteins (16, 40, 58, 133, 137, 155, 316).

Other adhesins identified in MG are GapA (or Mgc1) and Mgc2 (153, 169, 193). Like PvpA, the Mgc2 adhesin of MG localizes at the terminal tip structure of the cell surface (169). GapA is a primary cytadhesin that appears to work in a coordinated way with at least one other cytadherence-related protein,

CrmA undergoing concomitant phase variation in expression (153, 295, 318, 319, 321, 401). Expression of these two components has been correlated with binding to erythrocytes (401) and efficient attachment to cultured cells (318). These results demonstrated that both GapA and CrmA are required for MG cytadherence and pathogenesis (318, 320).

Some MG cytadhesin genes and proteins have homologues in other *Mycoplasma* spp., including some that are human pathogens, suggesting that there may be some conservation of cytadhesin genes and proteins among pathogenic mycoplasmas infecting widely divergent hosts (153, 169, 193, 277, 339).

Potent toxins have not been associated with mycoplasmas (328). See "Virulence Factors."

Strain Classification

Certain isolates of MG are known by their isolate or other designations and sometimes are called strains. Various strains of MG should not be confused with the numerous serotype designations that identified avian mycoplasmas prior to speciation within the genus *Mycoplasma*. Some MG isolates from both chickens and turkeys were described as "variant" or "atypical," because they were often difficult to isolate and less pathogenic, transmissible, and immunogenic than expected of field isolates (97, 198, 265, 412). Furthermore, because of demonstrated variability in antigenic phenotypes, MG strains, including well-established reference strains, may differ markedly in their antigen profiles and their virulence-related surface properties (340). Therefore, it has become increasingly important to develop methods to characterize and identify MG strains and strain variability. Recognition of intraspecies (strain) genotypic and phenotypic heterogeneity may be by serologic methods, or electrophoretic analysis of cell proteins or DNA, but has become more discriminatory and reproducible with the application of molecular techniques.

Antigenicity

Antigenic variation of MG strains and isolates has long been recognized empirically with reports of "atypical" or "variant" strains (97, 198, 412), and demonstrated by serologic assays (39, 97, 224, 251, 270, 316), immunoblots, and monoclonal antibodies (20, 58, 88, 133, 137, 175, 208, 241, 276, 316, 340, 353).

It is now well known that significant antigenic variability among MG strains can affect the sensitivity and specificity of serological tests, depending on the strain infecting the flock and the strain used to prepare antigen. Kleven *et al.* (224) tested MG strains with homologous and heterologous hemagglutination-inhibition (HI) assays and found that homologous HI titers were generally higher than heterologous titers. They also reported that MG strain A5969, commonly used as an HI antigen strain in many laboratories, was relatively insensitive for detecting antibodies against all of the strains studied and that none of the antigens tested was efficient in detecting HI antibodies against all other strains studied (224). Similarly, studies on the detection of antibody response to vaccination with MG strain ts-11 suggested that the major membrane antigen of MG had slightly different antigenic profiles in different strains, thereby necessitating the use of autologous (homologous) antigens in serodiagnostic assays to increase sensitivity of the tests for mycoplasma antibodies (305). Antigenic variability among MG strains presents a great challenge to the development and optimization of antigen/antibody-based assays (e.g., serologic, immunofluorescence, and growth inhibition organism identification tests).

The range of MG strain antigenicity, as well as its variability, is based at least in part on the organism's genomic commitment to immune evasion and adaptation to changes in the host environment, resulting in the expression of MG antigenic surface proteins that demonstrate high frequency variation, switching, and immune modulation (37, 39, 40, 133, 147, 240, 241, 255, 401). See "Antigenic Structure and Toxins," "Pathogenesis of the Infectious Process," "Immunity," and "Vaccination."

Immunogenicity or Protective Characteristics

As discussed previously, antigenic variability among and within MG strains and a range of immune responses to them have been recognized in the field and studied in the laboratory, culminating in current concepts of immune evasion, phenotypic switching, phase variation, etc. and the ongoing elucidation of the underlying mechanisms. See "Antigenic Structure and Toxins," "Pathogenesis of the Infectious Process," and "Immunity".

The immunogenicity (and relative low virulence) and protective characteristics of three MG strains (F, ts-11, and 6/85) have been applied to their commercial development as live vaccines (see "Live *M. gallisepticum* Vaccines"). Immunogenic and protective characteristics have also been described for some other MG strains that may be vaccine candidates including: house finch and house finch-like strains (122, 123, 311), and the GT5 strain (reconstituted from the avirulent R_{high} strain with the gene encoding the major cytadhesin GapA) (320). See "Pathogenesis of the Infectious Process," "Immunity," and "Vaccination."

Genetic or Molecular

M. gallisepticum strains have been differentiated from one another by direct comparison of protein banding patterns resulting from sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), or restriction fragment length polymorphism (RFLP) of DNA (206, 219, 224, 346). DNA and ribosomal RNA gene probes (204, 297, 418) and physical chromosomal mapping (380) have also been used to characterize MG intraspecies (strain) differences. These procedures for MG strain identification are costly, complex, and time consuming. Arbitrary primed PCR (AP-PCR) or random amplification of polymorphic DNA (RAPD) are polymerase chain reaction-based methods for MG strain identification that have proven efficient and very useful for epidemiological studies and for the identification of vaccine strains and field isolates (31, 73, 74, 75, 114, 117, 121, 144, 221, 244, 384). However, RAPD banding patterns are prone to variability and are difficult to reproduce and standardize, making interpretation challenging and subjective. More recently, *pvpA* or *mgc2* gene PCR followed by RFLP (PCR-RFLP) of the amplicon and/or sequence analysis has been used for MG strain identification (256, 263). Amplified fragment length polymorphism (AFLP) has been successfully used to explore the genomic variability of several *Mycoplasma* spp. including MG (75, 117, 172, 225, 349).

Molecular differentiation of MG by pulse-field electrophoresis (PFGE)(274), and targeted sequencing of various genes eg. *pvpA*, *gapA*, *mgc2* have also been reported (121, 172, 221).

Pathogenicity

Isolates and strains of MG vary widely in their relative pathogenicity, depending on the genotypic and phenotypic characteristics of the isolates, method of propagation, the number of passages through which they have been maintained, and the challenge route and dosage. Yolk-passaged organisms from MG-inoculated embryonated chicken eggs were often considered to be more infective than broth-passaged organisms.

The S6 strain of Zander (4, 112, 424) was an early pathogenic isolate from the brain of a turkey with infectious sinusitis. The A5969 strain was given that designation by Jungherr *et al.* (190) for a pathogenic culture supplied by Van Roekel. The R strain of MG was isolated by Dale Richey at the University of Georgia Poultry Disease (now Diagnostic) Research Center in 1963 from a chicken with airsacculitis. The R strain has been used widely for bacterin production and as a pathogenic strain for MG challenge studies (149, 214, 220, 338, 373, 416). The genotypic and phenotypic properties (including pathogenicity) of low (R_{low}) and high (R_{high}) passage R strains have been under intensive study, especially since the complete genome sequencing of R_{low} (319). R_{low} is capable of cytadherence, cell invasion, and is pathogenic while R_{high} shows diminished capacities in comparison (277, 320, 321, 402). Much *et al.* (294) reported that after aerosol inoculation of chickens, MG was re-isolated from the inner organs of birds infected with R_{low} but not recovered from the inner organs of birds infected with R_{high}. They concluded that these two mycoplasma populations derived from strain R differed in their capacity to cross the mucosal barrier, and that cell invasion may play a major role in the observed systemic spreading of MG (294).

The live F strain MG vaccine has proven relatively more pathogenic for turkeys than usually noted in chickens (243, 253, 338). The 6/85 and ts-ll vaccine strains are less pathogenic for chickens and turkeys then the F strain (1, 109, 110, 220, 269, 398, 399).

House finch and house finch-like strains of MG have shown relatively low pathogenicity for chickens and turkeys (122, 123, 311).

Clearly, there are MG strains that are primary pathogens for a number of avian host species. However, MG infections and diseases may be associated with a complex of host, environmental and microbial factors, so possibilities of multi-factorial etiologies and polymicrobial diseases involving MG should be considered. See "Pathogenesis of the Infectious Process".

Virulence Factors

Razin *et al.* (328) observed that the molecular mechanisms of mycoplasmal pathogenicity have remained largely elusive and that the clinical picture of mycoplasmal infections was more suggestive of damage due to host immune and inflammatory responses rather than to direct toxic effects by mycoplasmal cell components. Virulence factors associated with MG include motility and cytadhesion (24, 165, 209, 277, 287, 295); the ability to vary surface components, which may function in immune evasion and/or adaptation to host environments (37, 241, 271, 272, 273); and (possibly) the ability to invade cells (232, 294, 402). Papazisi *et al.* (319) sequenced the complete genome of MG strain R_{low} and identified a number of potential virulence factors involved in cytadherence, binding biomolecules, and heat shock proteins. Signature sequence mutagenesis, which allows for comprehensive screening of the MG genome for the identification of novel virulence-associated determinants from a mixed mutant population, was used to identify a virulence-associated gene encoding dihydrolipoamide dehydrogenase (174). See "Antigenic Structure and Toxins," "Pathogenesis of the Infectious Process," and "Immunity."

Pathobiology and Epidemiology

Incidence and Distribution

M. gallisepticum infections have resulted in important flock health and decision-making problems in chickens and turkeys in all areas of commercial production in the United States and are worldwide in distribution (223, 240).

In the United States, the incidence of MG in commercial poultry decreased considerably during the past 50 years, as the result of extensive control programs under the National Poultry Improvement Plan (NPIP) (14). The NPIP has been especially effective in controlling infections in primary and multiplier breeder flocks. However, outbreaks continue to occur in meat flocks, and MG infection is endemic in many large multiple-age commercial egg production units (see "Intervention Strategies"). There is increasing evidence that small backyard or "free-range/village" poultry flocks may be subclinically infected and sources of infection for commercial flocks (111, 194, 279, 377).

Beginning in 1994, MG was identified as the cause of conjunctivitis in free-ranging house finches and some other songbird species in the eastern United States and Canada (244, 245). The disease in house finches expanded to the bird's western range by 2002–05 (101, 248).

Natural and Experimental Hosts

M. gallisepticum infections naturally occur primarily in gallinaceous birds, particularly chickens and turkeys in commercial production. However, MG has also been isolated from naturally occurring infections in pheasants, chukar partridge, peafowl, bobwhite quail, and Japanese quail (41, 82, 296, 330). *M. gallisepticum* has also been isolated from ducks and geese (43, 61, 182), and from a yellow-naped Amazon parrot (47) and greater flamingos (105). *M. gallisepticum* was not isolated in culture but was detected by polymerase chain reaction (PCR) in 4 mature rooks (*Corvius frugilegus*) with pericarditis and pneumonia in a Scotland, UK survey of wild birds (323).

A report by Davidson *et al.* (92) of MG isolated from wild turkeys (*Meleagris gallopavo*) noted that the turkeys involved were in confinement, not free-ranging in their natural habitat. A follow-up survey of the same population 8 years later found no conclusive evidence that MG was present, indicating that MG did not persist or spread in this wild turkey population (260). Other surveys of wild turkeys have found MG sero-negative (173, 258)

and sero-positive (80, 130) populations. However, MG has rarely been isolated from wild turkeys, perhaps due in part to the common occurrence of other *Mycoplasma* spp., especially *M. gallopavonis* (80, 130).

Low prevalences (5 and 2.7%) of antibodies to MG were detected by serum plate agglutination (SPA) from lesser prairie-chickens sampled from southwestern Kansas USA in 2000 and 2001, but no confirmatory serologic or organism identification methods were used (158).

Prior to 1994, there were reports of mycoplasma isolations from various other free-ranging birds, but the significance of occasionally reported MG was not clearly established. Similarly, attempts to determine the pathogenicity of MG for various free-ranging birds were not very conclusive. However in 1994 free-ranging house finches (*Carpodacus mexicanus*) with peri-orbital swelling and conjunctivitis were observed in the mid-Atlantic and Eastern United States, and MG was isolated and shown to be the etiology (125, 226, 245, 259, 261, 365, 366). The disease quickly became widespread, affecting house finches throughout their entire eastern range and negatively impacting their population (94, 95, 162, 163, 304, 336). By 2002–05 the disease in house finches had extended to their western range (101, 248). *M. gallisepticum* conjunctivitis has been reported at much lower prevalence for American goldfinches, pine grosbeaks, and evening grosbeaks, with single confirmed isolations reported from a purple finch and blue jay (164, 244, 286). One field survey found tufted titmice positive by MG PCR but no isolates were made, and individuals from 10 additional songbird species were MG SPA positive only (262). In another field survey 358 birds representing 13 different families showed no clinical signs but 13 species from nine families had positive MG SPA reactions with all birds negative by PCR (116). Experimental infections produced clinical disease in pine siskins and tufted titmice, but house sparrows were infected without clinical disease (116).

Random amplified polymorphic DNA (RAPD) analyses of MG isolates spanning temporal, geographic and songbird species ranges resulted in banding patterns essentially identical to each other but different from other MG strains and isolates tested (244). These results indicated that this outbreak of MG in songbirds was caused by one and the same field strain (RAPD-type) and not by any vaccine strain (F, ts-11, 6/85) or isolate analyzed from poultry (244). More recent and further analyses (75, 324) showed some genomic variability among songbird MG isolates (see "Strain Classification") but supported previous observations that during the initial stages of the MG epidemic in songbirds, isolates from different geographic locations and songbird species had genotypes that appeared to be highly similar, further supporting a single point source of origin. One 2001 isolate from New York was clearly different from the other songbird samples and clustered together with the vaccine and reference strains, indicating that substantial molecular evolution or a separate introduction may have occurred (75).

Experimental infections using a house finch MG isolate found that infection and disease can result in chickens and turkeys but that biosecurity measures that restrict direct contact between poultry and infected house finches reduce the potential for transmission (311, 359). However, an MG isolate from commercial turkeys was very similar to isolates from house finches by RAPD and DNA sequence analyses suggesting the possibility that this was an incident of commercial poultry naturally infected by a songbird-like MG strain (122).

Specific-pathogen-free and/or mycoplasma-free domestic breeds of chickens, turkeys, and their embryonated eggs are the most commonly used experimental hosts for MG (49). Budgerigars, partridges, and house finches have also been used as experimental hosts (59, 116, 131, 226, 281, 335, 365, 366).

M. gallisepticum probably can infect susceptible birds at any age, although very young birds are seldom submitted with naturally occurring disease. However, young birds generally are considered somewhat more susceptible to experimental infections (49, 140).

Transmission, Carriers, and Vectors

Horizontal transmission occurs readily by direct or indirect contact of susceptible birds with infected clinical or subclinical birds resulting in high infection/disease prevalence within flocks. The upper respiratory tract and/or conjunctiva are portals of entry for the organism in aerosols or droplets (49). *M. gallisepticum* seldom survives for more than a few days outside of a host, so clinical or subclinical carrier birds are essential to the epidemiology of MG diseases. However, additional transmission and more widespread disease outbreaks may occur via fomites: contaminated airborne dust, droplets, or feathers, coupled with suboptimal biosecurity and personnel practices. Survival time of MG is dependent on at least conditions of substrate, pH, temperature and humidity. *M. gallisepticum* remained viable in chicken feces for 1–3 days at 20°C, on muslin cloth for 3 days at 20°C or 1 day at 37°C, and in egg yolk for 18 weeks at 37°C or 6 weeks at 20°C (70). *M. gallisepticum* survived in the human nasal passage for 24 hours; on straw, cotton, and rubber for 2 days; on human hair for 3 days; and on feathers for 2–4 days (78). Field isolates of MG did not survive longer than 7 days on paper discs at 30°C, 37°C and outdoor temperatures (298). Of 160 environmental samples tested from infected poultry flocks 103 were positive using MG specific PCR, but only 6 were positive by culture indicating that PCR is more sensitive for assessing dissemination of the organism than culture, which unlike PCR requires viable organisms for positive results (275).

Horizontal spread of MG within a chicken flock has been described in four phases: a latent phase (12–21 days) before antibody was first detected in inoculated birds; a period (1–21 days) in which infection gradually appeared in 5–10% of the population; a period (7–32 days) in which 90–95% of the remaining population developed antibody; a terminal phase (3–19 days) in which the remainder of the population became positive (282). Increasing the population density increased the rate at which horizontal spread occurred. Feberwee *et al.* (119) described an experimental model of horizontal transmission in chickens to study transmission dynamics and efficacy of intervention strategies.

Some potential reservoirs of MG carrier birds in the United States are backyard flocks (111, 279), multiple-age commercial layer flocks (215, 288), and some free-ranging songbird species

(122, 125, 244, 248, 286, 359). Good management and biosecurity practices are necessary to ensure that MG infections are not introduced from these and other sources to MG-free flocks.

Vertical transmission (*in ovo*/transovarian) of MG is known to occur in eggs laid by naturally infected hens (chickens or turkeys) and has been induced following experimental infections of susceptible leghorn chickens (152, 252, 314). The highest rates of transmission were found during the acute phase of the disease when MG levels in the respiratory tract peaked (151, 252). In separate studies, peak egg transmission was detected four weeks after MG challenge in approximately 25% of the eggs (152) and at three to six weeks post challenge in more than 50% of the eggs (347). On a flock basis, egg transmission rates decline as the postinfection interval lengthens. Transmission rates of approximately 3% at 8–15 weeks (334) and approximately 5% at 20–25 weeks (152) have been reported. During chronic infections under field conditions egg transmission is likely to occur at much lower levels (240). Horizontal transmission of MG from infected progeny that hatch, even very few infected birds resulting from very low transmission rates, is likely to involve entire flocks that receive any infected birds. *M. gallisepticum* control programs (14) must focus on primary and multiplier breeder flocks because of the severe epidemiological consequences of egg transmission. Serologic monitoring of breeder flocks at very short intervals (turkeys every 3 weeks, chickens every 2 weeks) could optimize the ability to detect and prevent the consequences of egg transmission.

Incubation Period

In experimental infections of chickens or turkeys, with uniform and high dosages, the MG incubation period varies from 6–21 days. Sinusitis often develops in experimentally inoculated turkeys within 6–10 days. However, development of clinical signs following a known exposure, even in turkeys that are considered highly susceptible, can be highly variable depending on MG strain virulence, complicating infections (polymicrobial infections), and environmental and other stressors (49, 97). Therefore under natural conditions it is very difficult to estimate the possible date of exposure based on the appearance of clinical signs. Many variable factors seem to influence the onset and extent of clinical disease so meaningful incubation periods cannot be stated. Chickens and turkeys often develop clinical infections near the onset of egg production, suggesting a low level of subclinical infection (perhaps due to egg transmission) that becomes clinical in response to stressors. This apparent extended incubation period was especially common in offspring of infected chickens or turkeys hatched from eggs dipped in antibiotic solutions for control of MG infection. The possible role of contamination from other (external) sources of infection is not always clear and can rarely be proved beyond reasonable doubt. Many outbreaks of MG appeared to represent this type of infection with delayed onset in which serologic evidence first appeared in older birds, often without clinical signs (265, 383, 412). In other situations, especially in turkeys exposed to virulent MG strains, clinical signs may be apparent before positive serology and are the first indicator of infection (249).

Clinical Signs

Chickens

The most characteristic signs of naturally occurring MG disease in adult flocks are tracheal rales, nasal discharge, and coughing. Feed consumption is reduced, and birds lose weight. In laying flocks, egg production declines but usually is maintained at a lowered level (288). However, flocks may have serologic evidence of infection with no obvious clinical signs, especially if they encountered the infection at a younger age and have partially recovered. Male birds may have the most pronounced signs, and the disease is often more severe during winter. In broiler flocks, most outbreaks occur after 4 weeks of age. Signs are frequently more marked than those observed in mature flocks. Severe outbreaks with high morbidity and mortality observed in broilers are frequently due to concurrent infections and environmental factors (216). See "Morbidity and Mortality."

Conjunctivitis developed in chickens following eye-drop inoculation of Australian field strains of MG combined with infectious bronchitis virus (356). Cases of keratoconjunctivitis caused by MG were reported in commercial layer chickens in Japan, first appearing around 30 days of age (310). Chickens showed swelling of the facial skin and the eyelids, increased lacrimation, congestion of conjunctival vessels, and respiratory rales.

Turkeys

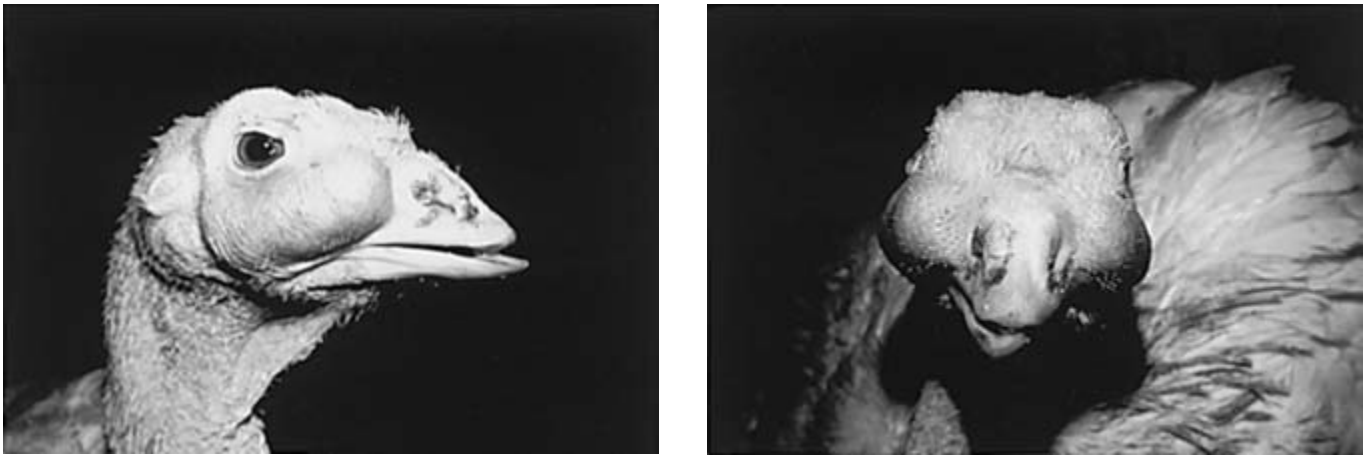
Turkeys are more susceptible to MG than chickens, commonly developing more severe clinical signs including sinusitis (Fig. 21.2), respiratory distress, depression, decreased feed intake, and weight loss. Inoculation by the eye-drop, intranasal, or intratracheal routes often results in fewer and milder lesions than intrasinus or intra-air sac inoculation (49). Turkeys sometimes do not develop sinusitis unless organisms are injected directly into the sinus (97). As in chickens, more severe outbreaks with high morbidity and mortality frequently result with the involvement of complicating factors such as colibacillosis or environmental stressors (216).

Nasal discharge with foamy eye secretions frequently precedes the more typical swelling of the paranasal (infraorbital) sinuses. Partial to complete closure of the eyes results sometimes from severe swelling of the sinuses. Feed consumption may remain near normal as long as the birds can see to eat. As the disease progresses, affected birds fail to gain weight and body mass may even decrease. Tracheal rales, coughing, and labored breathing may become evident if tracheitis or airsacculitis is present. An encephalitic form of MG has been reported in 12–16-week-old commercial meat turkeys displaying torticollis and opisthotonos (77). In breeding flocks, there may be a drop in egg production or at least lowered production efficiency.

Morbidity and Mortality

Embryos

Inoculation of broth cultures or exudates containing MG into 7-day-old embryonated chicken eggs via the yolk sac route usually results in embryo deaths within 5–7 days. One or more yolk passages may be necessary before typical deaths and lesions are produced. Dwarfing, generalized edema, liver necrosis, and en-



21.2. Turkey with advanced case of infectious sinusitis showing marked swelling of infraorbital sinuses and nasal exudate.

larged spleens are most typical. The organism reaches its highest concentration in the yolk sac, yolk, and chorioallantoic membrane (CAM) just prior to embryo death. Studies showed that MG strains varied in their *in ovo* pathogenicity and that there was no correlation between *in ovo* pathogenicity and other *in vivo* or *in vitro* methods for pathogenicity evaluation (239). *M. gallisepticum* embryo mortality was prevented in eggs containing maternal MG antibodies, although MG could be re-isolated from the yolk sac membrane of live embryonated eggs after 17 days of incubation (239). Inoculation of embryonated eggs is rarely employed for the primary isolation of avian mycoplasmas now that adequate culture media are available.

Chickens

M. gallisepticum infection usually affects nearly all chickens in a flock but disease is variable in severity and duration. It tends to be more severe and of longer duration in the cold months and affects younger birds more severely than mature birds, although there may be a considerable loss from lowered egg production in laying flocks.

Although MG is considered the primary cause of chronic respiratory disease (CRD), other organisms frequently cause complications. Severe air sac infection, frequently designated as complicated CRD, or air sac disease, is the condition more commonly encountered in the field. Newcastle disease (ND) or infectious bronchitis (IB) may precipitate outbreaks of MG infection. *E. coli* is a frequent complicating organism. The effects of MG, *E. coli*, and IB virus (IBV) infections alone or together in chickens have been reported (113, 156, 157, 125). Severe air sac infection was reproduced when all three agents were combined, and *E. coli* did not readily infect the air sacs without previous infection by MG alone or in combination with either IBV or ND virus. Investigators have noted increased severity and duration of the disease when both MG and IBV were present (356).

Mortality may be negligible in adult laying flocks, but there can be a reduction in egg production (68, 288). In broilers the mortality may range from low in uncomplicated disease to as much as 30% in complicated outbreaks, especially during the colder months. Retarded growth, downgrading of carcasses, and condemnations constitute further losses.

Turkeys

M. gallisepticum infection of turkeys causes disease in most of a flock, although turkeys may not exhibit sinusitis and the lower respiratory form of infection may be most prominent (97). Infection with MG may last for months in untreated flocks. Clinical signs, morbidity, and mortality associated with MG infection in turkeys may be highly variable. Typically, meat turkeys experience outbreaks between 8–15 weeks of age. Initially, mild respiratory signs may progress in 2–7 days to a severe cough in 80–90% of the flock. Swollen sinuses with nasal discharge may affect 1–70% of birds in affected flocks. Condemnations primarily result from airsacculitis and related systemic effects.

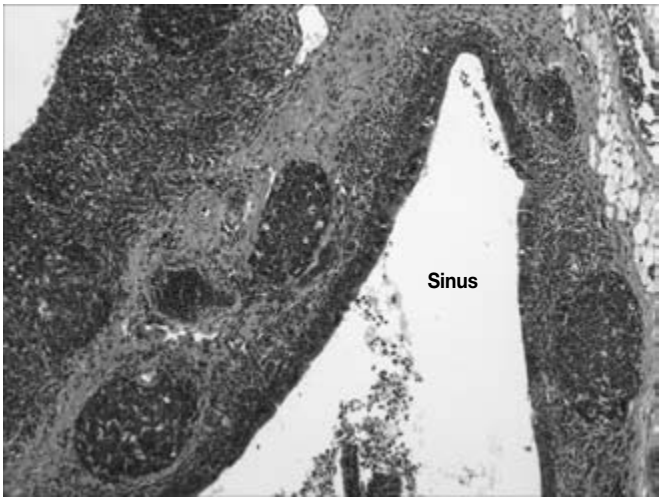
Pathology

Gross

Gross lesions consist primarily of catarrhal exudate in nasal and paranasal passages, trachea, bronchi, and air sacs. Sinusitis is usually most prominent in turkeys but is also observed in chickens and other affected avian hosts. Air sacs frequently contain caseous exudate, although they may present only a beaded or lymphofollicular appearance. Some degree of pneumonia may be observed. In severe cases of typical air sac disease in chickens or turkeys, there is the triad of airsacculitis, fibrinous or fibrinopurulent perihepatitis, and adhesive pericarditis resulting in high mortality and extensive condemnations at processing. However, these lesions may occur with chlamydiosis or septicemia and are not pathognomonic for MG. Commercial layer chickens with MG keratoconjunctivitis had marked edema in the facial subcutis and eyelids, with occasional corneal opacity (310). Conjunctivitis with periorbital swelling and inflammation are characteristics of MG in house finches and other songbirds (244, 245, 286) and has been seen in chukar partridges (281). Oviducts distended with exudate (salpingitis) have been associated with decreased egg production in MG-infected flocks (100, 308).



21.3. This cross-section of the nasal cavity shows the infraorbital sinus and nasolacrimal duct. The mucosa of each is increased in thickness due to a nodular and diffuse infiltration of lymphocytes and other lymphoid cells. The increase in number of lymphoid nodules is called the *lymphofollicular reaction*.



21.4. The epithelium of the infraorbital sinus (Sinus) is increased in thickness, and there is a nodular proliferation of lymphoid cells in the connective tissue beneath the lining epithelium. The mucosa of the nasolacrimal duct is increased in thickness due to the lymphoid cell infiltration. Exudate is in the sinus.

Microscopic

Microscopic pathology of MG in chickens and turkeys is characterized by marked thickening of the mucous membranes of affected tissues from infiltration with mononuclear cells and hyperplasia of the mucous glands (72, 168, 388) (Fig. 21.3). Focal areas of lymphoid hyperplasia are commonly found in the submucosa (Fig. 21.4). In trachea, an almost complete destruction of cilia was observed and swollen epithelial cells with MG adhering to villi (72, 102) 81). In tracheal ring cultures, ciliostasis resulted from MG infection (354, 369, 385). Tracheal gross and microscopic lesions, particularly increased mucosal thickness, have been used as measures of MG infection and disease (309, 397). Following experimental infection of chickens, trachea mucosal thickness increased significantly from 1–2 weeks and then decreased from 2–3 weeks (139). Lungs have pneumonic areas, lymphofollicular changes and granulomatous lesions. Detailed examinations of MG-infected chicken air sacs via light microscopy, scanning EM, and histomorphometric evaluation have been reported (382).

Keratoconjunctivitis in layer chickens associated with MG was characterized by epithelial hyperplasia, severe cellular infiltration, and edema of the subepithelial and central fibrovascular connective tissue stroma, which resulted in marked thickening of the eyelids (310). In the subepithelial lamina propria, proliferation of plasma cells and lymphocytes accompanying germinal centers was marked and resulted in irregular elevations of the overlying hyperplastic epithelial layer (310).

Histologic examination of turkey brains in cases of encephalitic MG revealed moderate to severe encephalitis with lymphocytic cuffing of vessels, fibrinoid vasculitis, focal parenchymal necrosis, and meningitis (77).

Salpingitis associated with reduced egg production in layer chickens was characterized by marked thickening of the oviductal mucosa due to epithelial hyperplasia and marked lymphoplasmacytic infiltration (308).

Ultrastructural

Ultrastructural details of MG interaction with the tracheal epithelium of chickens *in vivo* and *in vitro* have been reported (2, 102, 170, 229, 367). Tracheal lesions occurred in close association with the presence of mycoplasmas and were characterized by degeneration of the epithelial cells and inflammatory cellular infiltration of the mucosa (367). Mycoplasmas were predominantly found extracellularly and only rarely in phagocytic vacuoles of epithelial cells. Mycoplasmas were attached to epithelial cells by their terminal organelles (blebs or tip structures) close to the host cell membrane. A release of mucous granules followed by exfoliation of ciliated and non-ciliated epithelial cells and infrequent loss of cilia from individual cells was noted (102). Repair of the epithelial surface was affected by basilar epithelial cells differentiating and filling in the spaces formed by exfoliated cells. During infection, there was increasing epithelial thickness due to cellular infiltration and edema (102).

Incubation of MG with chicken red blood cells resulted in alterations of cell surface morphology and perforations (228, 230); results that may support recent evidence that MG can penetrate and be present within cells (402).

Pathogenesis of the Infectious Process

Except for infections acquired by egg transmission, the upper respiratory tract and/or conjunctiva generally are accepted to be the portals of entry for naturally acquired MG infections. *M. gal-*

lisepticum is considered to be primarily a surface parasite of the respiratory tract and conjunctiva, although spread to other organs (e.g., brain (77, 378)) indicates that transient systemic infections occur, resulting in acute and chronic diseases at multiple sites. Oviduct infections (308, 325) may result from proximity to infected air sacs (334). Attachment of mycoplasmas to host cells is a prerequisite for successful colonization, infection, and pathogenesis (91, 153, 237, 295, 327) and is considered an important virulence factor (see "Virulence Factors" and "Strain Classification/ Antigenicity"). *M. gallisepticum* organisms attach to epithelial cells by their terminal organelle (bleb or tip structure) which also plays a role in gliding motility, another component of pathogenesis (69, 350, 367). Comparative studies using MG strains R_{low} (virulent) and R_{high} (avirulent) identified the cytadhesin protein GapA and accessory protein CrmA, and found that both are required for cytadherence and pathogenesis (295, 318, 321), and that phase variation of these proteins correlates with hemadsorption phenotypic switching (401).

In tissue culture, MG was found capable of entering non-phagocytic host cells, a property that could provide the organism with the opportunity for resisting host defenses and selective antibiotic therapy, establishing chronic infections, and passing through the respiratory mucosal barrier to cause systemic infections (402). Much *et al.* (294) found that R_{low} and R_{high} strains differed markedly in their ability to invade non-phagocytic eukaryotic cells, and after aerosol inoculation of chickens MG was re-isolated from the inner organs of birds infected with R_{low} but not R_{high}. Results showed that these strains differed in their capacity to cross the mucosal barrier, and suggested that cell invasion may play a major role in the systemic spread of MG and avoidance of host defenses allowing for its survival and persistence of infection (294). Furthermore, since adhesion is a prerequisite for cell invasion, the lack of GapA expression in R_{high} may account for its inability to cause systemic infection and air sac lesions (294).

Changes in tracheal epithelial surfaces induced by MG infection *in vivo* and *in vitro* included release of mucous granules followed by destruction and exfoliation of ciliated and nonciliated epithelial cells (72, 102). In chicken embryos, MG infection resulted in extensive deciliation, surface erosion, and inflammatory cell infiltration (229). These changes, and ciliostasis as noted in tracheal organ cultures infected with MG (369, 385), likely play both primary and secondary roles in the pathogenesis of MG diseases.

Suppression or stimulation of B and T lymphocytes and cytokine induction (139, 231, 328) may also play roles in pathogenesis. A prominent feature of MG infection is a lymphoproliferative response at the site of infection, and MG-infected cells have been shown to produce chemotactic factors attracting the migration of heterophils and lymphocytes (227, 233). Incubation of MG with chicken red blood cells (RBC) resulted in altered cell surface morphology, decreased cell size, and perforations of the cells, suggesting that cell penetration may occur and that RBC damage may result in pathologic consequences (228, 230).

In the absence of exacerbating environmental stressors or infection with other pathogens, MG infection in the trachea may be self-limiting (406) but organisms may persist (carrier state) even in the presence of humoral or local antibody (39, 214, 406). The high frequency phenotypic variation of major surface antigens (133, 147, 401) and cell invasion (294) may be explanations for MG chronic infections, despite a strong immune response (240) (see "Immunity").

Complicating infections (polymicrobial disease), especially colibacillosis, and including some live vaccines, are known to result in more severe MG diseases (157, 181, 216, 299, 302). Other factors that are likely to contribute to more severe MG diseases include immunosuppression and poor environmental conditions or other stressors (34).

Immunity

Chickens or turkeys that have recovered from clinical signs of MG diseases are known to have some degree of immunity. However, recovered birds may still carry the organism (39) and can transmit infections to susceptible birds by contact or egg transmission. The early literature concerning the immune response to MG was reviewed by Luginbuhl *et al.* (257). The importance of antibodies and the bursa of Fabricius to the development of MG resistance and serologic response to the organism has been demonstrated (3, 179, 234). However, poor correlation between levels of specific circulating antibody and protection has also been well documented (234, 254, 306, 373, 398). Increasing antibody titers to MG were found in tracheal washings of infected chickens with a concomitant decrease in organisms and tracheal lesion scores (76, 406). Antibodies persisted in recovered chickens, and upon re-exposure, they had a faster rate of MG elimination and less severe tracheal lesions than observed after the first exposure. These results and others indicated that antibodies in respiratory secretions played a role in resistance to MG (22, 106, 179, 404, 406). Respiratory tract antibodies produced in response to MG infection inhibited attachment of the organism to tracheal epithelial cells (22), which may be one important mechanism of immune-mediated protection. The presence of maternal antibodies to MG in embryonated eggs reduced the *in ovo* pathogenicity of infection and increased the probability of survival of infected embryos (42, 239, 254). Considerable effort has been made to identify MG antigens, especially those with cytadhesion properties, which may play key roles in the pathogenesis of, and immune response to, infection. See "Antigenic Structure and Toxins," "Virulence Factors", and "Vaccination."

Mycoplasmas may affect the cell-mediated immune system by inducing either suppression or stimulation of B and T lymphocytes, and inducing cytokines (76, 132, 139, 231, 328, 329). Lymphoproliferation, interferon, and nitric oxide were detected *in vitro* in antigen-stimulated peripheral blood leukocytes from MG-infected chickens (329). Gaunson *et al.* (139, 141) examined the numbers and distribution of lymphocytes in the tracheas of chickens exposed to virulent and vaccine strains of MG and found specific stimulation of CD8+ cells particularly in the acute phase of disease. They observed a primary role for local antibody mediated responses in controlling MG infection, but also presented evidence for significant natural killer and cytotoxic T cell responses to infection (141).

Studies on variable expression of MG immunodominant surface proteins demonstrating high frequency variation, switching, and immune modulation suggest that this variability may function as a crucial adaptive mechanism enabling the organism to escape from the host immune defenses (immune evasion), adapt to the changing host environment at different stages of a natural infection, and allow for chronic infection despite a strong immune response (39, 40, 133, 147, 240, 241, 401). Additionally, it may be possible for MG to hide from host defenses by entering eukarytotic cells (cell invasion) and spreading systemically, contributing to its survival and persistence of infection (228, 230, 294, 402).

Diagnosis

Isolation and Identification of Causative Agent

The gold standard for MG diagnosis is isolation and identification of the organism. To culture MG, suspensions of tracheal or air sac exudates, turbinates, lungs, or fluid sinus exudate should be inoculated directly to mycoplasma broth or agar medium (217). Swabs can also be taken from the trachea or choanal cleft (palatine fissure) for MG culture (57, 421, 422). *M. gallisepticum* may also be present in oviducts (100, 308), and has been isolated from the cloaca of turkeys and chickens (9, 264, 390). For culture medium and isolation methods, see "Growth Requirements" and Kleven (217).

During the acute stages of infection (generally in the first 4–8 weeks postinfection), the population numbers of MG in the upper respirator tract and the prevalence of infection in the flock are high (223, 238, 406). Therefore, swabbing tracheas or choanal clefts of 10–20 live birds is usually sufficient to recover the organism, whereas 30–100 cultures may be required at later stages (223). Tracheal or choanal numbers of organisms in chronically infected birds, such as commercial egg layers or backyard poultry, may be so low that MG organism may not be detected by usual sampling and culture methods (240). To optimize the possibility of isolation, flocks should be sampled for MG culture prior to initiation of antimicrobial therapy (285). Ammonium chloride (54) and perhaps other drinking water treatments may hinder the isolation of MG from infected birds.

The following recommendations are for specimens that must be stored and/or shipped to a laboratory for culture (223). Ideally, mycoplasma broth media should be inoculated directly from the bird (swab, exudate, or small tissue sample) and incubated (37°C) immediately. If short-term storage and/or shipment are necessary, keep inoculated broths at 4°C or on cold packs (not longer than 24 hours) and use an overnight carrier. Alternatively, tracheas, fluids, or tissues can be collected and frozen on dry ice but must remain on dry ice until arrival at the laboratory.

Samples of growing broth cultures are transferred to mycoplasma agar medium to allow for colony formation followed by immunofluorescence tests for *Mycoplasma* spp. identification. Direct or indirect immunofluorescence of mycoplasma colonies or colony imprints (138) are commonly used to identify MG field isolates, even with cultures of mixed *Mycoplasma* spp. (217, 223, 292, 371, 372). Immunoperoxidase procedures, either alone or in combination with immunofluorescence, may also be used for rapid identification of mycoplasma cultures (38). Growth inhibition has also been used for *Mycoplasma* spp. identification (79, 217). Mycoplasma species-specific hyperimmune sera, usually produced by research laboratories in rabbits or avian species, are essential reagents for these diagnostic tests (217, 223).

Inoculation of 7-day-old embryonated chicken eggs via the yolk sac with exudates or suspensions from suspect lesions may be used as another means of isolating MG, but the inocula must be free from bacterial and fungal contamination (49, 104, 333). Death of embryos should occur within 5–8 days, but one or more serial passages of harvested yolk material might be required before embryo deaths and typical lesions are noted.

Detection of MG using DNA and ribosomal RNA gene probes has been described (99, 124, 135, 143, 176, 205, 345), but for most applications these methods have been superseded by various polymerase chain reaction (PCR) based procedures that are relatively less complex and more rapid, sensitive, and specific (115, 136, 161, 196, 300, 343, 344, 352, 355, 392). Multiplex PCR protocols have been described which allow for the simultaneous detection of different organisms (267, 317, 392). A test based on PCR of the 16S rRNA gene with *Mycoplasma*-specific primers and separation of the PCR product according to primary sequence using denaturing gradient gel electrophoresis has been described (278). Rapid detection by real-time PCR has also been described (67, 283).

Detection of MG-specific DNA by PCR has become widely available at diagnostic and institutional laboratories using commercial PCR kits or established protocols (134, 235). Detection of MG DNA by PCR compared to isolation of the organism in culture provides a negative or positive result in hours instead of days, does not rely on the presence of viable organisms, and is much less susceptible to microbial contaminants. However, culture and isolation of MG organisms remains essential for most further studies such as experimental infections, pathogenicity studies, and intra-species (strain) identification (see "Strain Classification"). An inoculated mycoplasma broth can be divided and processed for both culture and PCR. When culture and isolation of viable organisms is not necessary or possible, FTA® filter paper may be used for the inactivation and storage of MG suspensions or field specimens prior to PCR or other DNA-dependent assays (293).

Serology

Serologic procedures are useful for flock monitoring in MG control programs (14) and to aid in diagnosis when infection is suspected. A positive serologic test together with history and clinical signs typical of MG disease allows a presumptive diagnosis pending isolation and/or identification of the organisms.

The tube agglutination test was a common procedure, especially during the MG control program for turkeys in the 1960s and 1970s but is now rarely used. Serum plate agglutination (SPA) antigen, for the detection of antibodies to MG, is commercially available. Because the SPA test is quick, relatively inexpensive, and sensitive, it has been widely used as an initial screening test for flock monitoring and serodiagnosis (14, 217, 223).

However, nonspecific reactors occur in some flocks infected with *M. synoviae* due to cross-reactive antigens (18, 36), or in flocks recently vaccinated with oil-emulsion vaccines and/or vaccines of tissue-culture origin against various agents (5, 150, 341, 414). Certain nonspecific SPA reactions may be reduced by diluting the test serum (341). Some laboratories determine agglutination end points by preparing 2-fold (1:2) dilutions of sera in saline. Sera that react at 1:8 or greater are considered positive, which may also be useful in differentiating between specific and nonspecific reactions (217). The SPA test is highly efficient in detecting IgM antibody, which is the first class of immunoglobulins produced in response to infection (211).

The hemagglutination-inhibition (HI) test has been commonly used to confirm reactors detected by SPA or, more recently, enzyme-linked immunosorbent assays (ELISA). However, the HI test is time consuming, the reagents are not commercially available, and the test may lack sensitivity (97, 217, 223, 224).

Enzyme-linked immunosorbent assays were developed to increase testing efficiency, and improve sensitivity and specificity of results relative to the SPA and HI tests (19, 88, 89, 166, 266, 290, 362, 374). Commercial ELISA test kits are now commonly used for flock monitoring and serodiagnosis. In general, ELISA tests may be slightly less sensitive but more specific than SPA tests and less specific but more sensitive than HI tests (19, 111, 192, 197, 200). In many production company and diagnostic laboratories, ELISA is the test of choice for MG serology. Efforts continue to improve MG ELISA sensitivity and specificity by identifying and purifying specific immunodominant MG proteins for use as ELISA antigens (20, 88, 108, 305, 313, 357). ELISAs have also been used to detect MG antibodies in respiratory tract washings (22, 406) and egg yolk samples (197, 289). Studies comparing egg yolk and serum for the detection of MG antibodies by ELISA or HI found comparable results, indicating that egg yolk samples could be used instead of serum samples for flock screening (60, 197, 289). Dot immunobinding assays for the detection of MG antibodies have also been described (17, 85, 86, 370).

Positive MG serologic test results have been encountered in some chicken breeding flocks with histories of being MG-free. The usually normal-appearing flocks began to have a small percentage of MG SPA test reactors at 28–36 weeks of age. Hemagglutination-inhibition titers rarely exceeded 1:80, and the percent of SPA reactors often did not exceed 20–40% of the flock during several months of study (265, 412). Low virulence MG strains, which were difficult to isolate and apparently egg transmitted, were considered responsible (383, 412). Turkeys have also been infected with MG isolates of low virulence, low transmissibility, and poor immunogenicity (97). Antigenic variation of MG isolates demonstrated by immunoblots (20, 23, 353) and agglutination (316) or HI assays (97, 224, 270) is at least partly responsible for "atypical" reactors.

The use of certain antimicrobics, especially early in the course of infection, may effect the development of a detectable antibody response (240, 360). In experimental infection trials, fewer serological responses were found in MG-infected chickens or turkeys treated with antibiotics than in the MG-infected nonmedicated groups (184, 187, 189, 285).

The performance of culture, PCR, plate agglutination, HI, and ELISA tests were compared for the detection of MG infection using samples from SPF layer chickens infected at 66 weeks of age, and the authors concluded that it was not advisable to rely completely on only one test system (118).

Differential Diagnosis

M. gallisepticum infections of poultry must be differentiated from other respiratory diseases and may be clinically obvious only when present in conjunction with complicating respiratory infections such as Newcastle disease or infectious bronchitis and *E. coli* (216, 217).

In chickens care must be taken to differentiate MG infection from other common respiratory diseases. Newcastle disease and infectious bronchitis or their antibodies may be present as separate entities or as part of the complicated CRD syndrome. Infectious coryza (*Avibacterium paragallinarum*) and fowl cholera (*Pasteurella multocida*) usually can be identified by bacterial culture. *M. synoviae* infection may be present alone or in addition to MG. Application of both serologic and organism identification test procedures may be necessary in some cases.

In turkeys respiratory disease including sinusitis may be due to avian influenza, aspergillosis, pasteurellosis, chlamydiosis, respiratory cryptosporidiosis, Newcastle disease, MS infection, or vitamin A deficiency as well as MG (217). Avian pneumoviruses (APV) and *Ornithobacterium rhinotracheale* (ORT) should also be considered in the differential diagnosis (180, 387). Specific organism identification and/or serologic procedures are needed to differentiate MG from other microbial causes of turkey respiratory disease.

In songbirds with conjunctivitis, *M. sturni* (127, 128, 246, 323, 394) (isolated from European starling, northern mockingbird, blue jay, crows, blackbird, magpie, American robin) should be considered as well as MG (125, 163, 164, 244, 286) (isolated from house finch, American goldfinch, purple finch, blue jay, evening grosbeak, and pine grosbeak). Chlamydiosis, other bacterial infections, and poxvirus should also be considered.

A mycoplasma strain designated 4229T, isolated in 1984 from the turbinate of a duck in France, and similar isolates from geese in France and from a partridge in England, were originally identified as MG by immunofluorescence and growth inhibition tests (48). Subsequent serologic and molecular studies indicated only a partial relationship to MG, and DNA-DNA hybridization studies revealed only approximately 40–46% genetic homology (48). A new species name, *Mycoplasma imitans*, was proposed for this organism that cross-reacts serologically with MG (48, 339) but can be differentiated by molecular techniques (134, 161, 195, 343). *M. imitans* has not been identified in the United States.

Intervention Strategies

Management Procedures

Because MG can be egg transmitted, maintaining chicken and turkey flocks free of MG infection is only possible by starting with breeding stocks that are known to be free of the infection and then rearing them with adequate biosecurity to avoid intro-

duction of the organism. Establishing the MG-clean status of breeder flocks and maintaining that status can be accomplished by participation in control programs. In the United States, turkey and chicken primary and multiplier breeders and hatcheries generally have adopted the various MG control programs of the National Poultry Improvement Plan (14) with great success. However, multiple-age production complexes, usually for commercial table egg production, but also for breeders and meat flocks, have become more common worldwide (240). Poultry population densities have increased, sometimes involving multiple companies and different poultry types (breeders, meat birds, layers), in close proximity to each other and backyard flocks. Perhaps because of these risk factors and lapses in biosecurity, maintaining MG-free poultry flocks may be difficult or impossible (240). Therefore, appropriate antimicrobial therapy may be used to reduce morbidity and mortality, losses at processing, or egg transmission. Vaccination may also be an option in some situations.

Vaccination

Types of Vaccines

Nonliving M. gallisepticum Vaccines. Interest in MG vaccines originated in the late 1970s as it became apparent that MG infection was endemic in some multiple-age, egg-laying complexes. *M. gallisepticum* bacterins (killed organisms) with oil-emulsion adjuvant protected young chickens from intra-sinus challenge with virulent MG and commercial egg layers from MG-induced drops in egg production (167). Some investigators found that bacterins could protect broilers from airsacculitis (191, 417) or layers from reductions in egg production (416), and others did not detect much efficacy in commercial egg layers with endemic MG infections (207). Bacterins have been shown to reduce, but usually not eliminate, colonization by MG following challenge (214, 373, 404, 416, 417) and generally are felt to be of minimal value in long-term control of infection on multiple-age production sites (240). A study on the effect of bacterin vaccination on the horizontal transmission of MG in a model system found that there was some reduction in shedding, but concluded that bacterin vaccination would not reduce horizontal transmission of MG between laying hens (120).

Because bacterins are nonliving, they do not engender the safety concerns of live vaccines; however, they are disadvantaged by the need for more than one application (for optimal protection) and the cost of individual bird administration. To enhance the performance of nonliving MG vaccines, various adjuvants and antigen delivery systems, including liposomes and iota carrageenan, have been investigated (27, 28, 30, 106, 107, 405). *M gallisepticum* bacterins have been produced commercially.

Development of subunit vaccines using MG surface proteins has been investigated (90, 280, 342, 364, 420) and may be gaining interest as an additional alternative to bacterins and live vaccines.

Live M. gallisepticum Vaccines. Live F strain MG vaccine is a relatively mild strain that apparently originated from the Connecticut F strain (386), and has been used in pullet immunization programs (68, 151, 338). However, the original F strain isolate was described as a typical pathogenic strain (407). That strain was used in Connecticut for live culture vaccination of young broiler breeder replacement flocks to reduce possible egg transmission of MG in subsequent breeder flocks (257). Studies using the Connecticut F strain in young replacement pullets prior to housing in multiple-age egg-laying complexes have also been reported (68, 386). Numerous reports exist on the use of live F strain MG vaccine, which has been produced commercially and used extensively in multiple-age laying complexes to reduce MG-caused egg production losses (1, 53, 55, 68, 83, 87, 151, 152, 212). In broilers, vaccination with F strain provided some protection from airsacculitis following aerosol challenge with virulent R strain (238, 337, 338). The biologic mechanism underlying protection by F strain did not involve competition for adherence sites or blockage by prior colonization, and F strain vaccination did not prevent colonization by the challenge strain of MG (238). F strain can be transmitted through the egg (252) and among pen-mates (109, 212). However, pullets given F strain by the eye-drop route did not transmit the infection to broilers in pens in the same house when separated by an aisle or empty pen (212). F strain vaccinated laying hens produced more eggs than unvaccinated hens in flocks with endemic MG but not as many as MG-clean flocks (68, 288). Vaccination of pullets in isolation facilities at twelve weeks of age resulted in delayed onset of lay and decreased total egg production (62). A series of trials using this model system followed possible physiological mechanisms involved in the effects of F strain vaccination on reproductive performance (63, 64, 65, 66, 322). F strain vaccinated flocks maintain the organism in the upper respiratory tract for the life of the flock (212). In laboratory studies F strain vaccination reduced the population of a challenge strain in the upper respiratory tract (83, 238); in pen trials F strain vaccination effectively displaced infection with a challenge strain (220). Field-strain MG was displaced from a multiple-age layer complex following 2 years continuous use of F strain vaccine in replacement pullets (222). Following experimental infection F strain was found to be pathogenic in turkeys (253), and it has been associated with MG outbreaks in breeder and meat turkeys under field conditions (243). F strain vaccine can be administered by several routes including eye-drop, intranasal, and coarse spray (240). Vaccine generally is administered at 8–14 weeks of age but can be administered as early as 2 weeks or less if chicks are at risk of exposure to wild-type infection before 8 weeks (240).

The 6/85 strain of MG originated in the United States, and its development and vaccine characteristics have been described (52, 109, 110). Studies using MG 6/85 vaccine found minimal virulence in chickens and turkeys, little or no transmissibility from bird to bird, and resistance against challenge with virulent MG (1, 109, 221, 247, 423). The 6/85 vaccine is administered by spray, results in little or no detectable serologic response, and can be detected in the upper respiratory tract for 4–8 weeks after vaccination (1, 109, 247). In the United Sates, MG 6/85 vaccine has been used primarily for the prevention of egg production losses in commercial table-egg layers. This vaccine is formulated as a freeze-dried pellet and administered as a single dose to pullets 6

weeks of age or older. To be fully effective, the vaccine must be administered by the aerosol route.

Development and characterization of the ts-11 MG vaccine have been described (398, 399). The ts-11 vaccine originated from an Australian MG field isolate (strain 80083) that was subjected to chemical mutagenesis and selected for temperature-sensitivity (growth at 33°C) (399). The ts-11 MG vaccine has minimal or no virulence for chickens and turkeys, is weakly transmissible from bird to bird, stimulates a slow to develop response and low levels of detectable circulating antibody, and induces protection to MG experimental and field challenge (1, 26, 45, 140, 247, 306, 396, 398, 399). Detection of antibody response to ts-11 vaccination may be improved by using an ELISA test with autologous antigen (305). In vaccinated flocks ts-11 persists for the life of the flock in the upper respiratory tract and induces long-lived immunity (396). In experimental studies vaccination of pullets had no impact on egg production, egg and eggshell quality parameters, and egg size distribution (56). Experimental vaccination of broiler breeders showed resistance to infection by field MG in the breeders and their embryos, and better production performance in their broilers (26). Floor pen studies used to evaluate the serological response of broiler breeders after vaccination with ts-11 (81) showed a pattern of seroconversion that was different from results obtained with layer chickens (1, 247, 398) (there were other experimental differences as well). Lateral spread of ts-ll to commingled pen-mates occurred rapidly, causing seroconversion patterns to mimic those of vaccinated pen-mates (81). In the United Sates, MG ts-11 vaccine has been used primarily for prevention of egg production losses in commercial table egg layers. The vaccine is formulated as a frozen (–40°C) suspension and administered by the eye-drop route as a single dose to growing pullets 9 weeks of age or older, at least 3 weeks before expected exposure to field challenge. However, Gauson *et al.* (140) reported that ts-11 vaccine was safe in birds between 1 and 4 weeks of age and effective in preventing development of severe disease in them after challenge, even though birds of the same age were more susceptible to development of disease when infected with virulent MG.

Comparative studies and reviews on experimental and field uses of MG vaccines in poultry are available and should be consulted for more details relative to the summaries that follow. Because of their superior safety characteristics (relative avirulence and low potential for unintended transmission to non-target e.g. unvaccinated flocks), both 6/85 and ts-11 vaccines may be considered preferable to F strain when MG vaccination is necessary in situations where susceptible poultry are nearby (240). An important characteristic of MG vaccines is the ability to induce resistance to infection from wild-type challenge, resulting in displacement of wild-type strains with the vaccine strain on multiple-age production sites (218, 240). This characteristic could be used as a tool for MG eradication from such sites. F strain vaccine had greater ability to displace challenge strains in pen trials than did 6/85 or ts-11, and displaced a field strain of MG on a multiple-age commercial egg production site (218, 222). However, when vaccination on this farm was stopped, F strain continued to cycle from flock to flock, and eradication of MG was not achieved (222, 240). The ts-11 vaccine was used to vaccinate replacement pullets on a site previously populated with F strain, and displacement of F strain by ts-11 occurred in the vaccinated flocks. After vaccination with ts-11 was discontinued, MG was no longer detected on the farm (240, 384). Similar data for the 6/85 vaccine are not available, but there are reports of complexes that have used 6/85 and became seronegative, suggesting that displacement of wild-type MG strains may have occurred (218, 240). If wild-type MG strains are highly virulent, it may be necessary to vaccinate with F strain for one or more production cycles prior to switching to either 6/85 or ts-11 (218, 240).

Because they are live vaccines, there are concerns for the safety of F, 6/85, and ts-11 strains relative to non-target flocks that relate mainly to virulence, persistence, transmissibility, and the stability of these vaccine properties. The known biological characteristics of F strain vaccine are compatible with examples of "escaped" F strain vaccine associated with MG infection and/or disease in nontarget flocks (218, 243, 253). The 6/85 vaccine has generally been very safe for chickens. However, there have been several instances of 6/85-like isolates (based primarily on RAPD genotyping) made from turkeys showing clinical disease and associated in most cases with a history of chickens vaccinated nearby (218, 221). A genotypically 6/85-like isolate was made from unvaccinated table-egg layers with swollen sinuses (379). The isolate had some phenotypic properties unlike 6/85 vaccine, and the origin and role of the 6/85-like isolate was not established (379). However, it was recommended that live MG vaccines be properly administered so that all birds in the target flock are properly immunized; and that unvaccinated, susceptible flocks should not be allowed to remain in close proximity to vaccinated flocks (379). Isolates that were ts-11-like have been detected on at least two occasions in unvaccinated chicken flocks (218). In both instances, there was a history of possible inadvertent vaccination, and in one there was subsequent spread to neighboring broiler breeders (218). These experiences suggest that even though these vaccines are generally very safe, they may have the potential for infecting non-target flocks (218). Live MG vaccines should be used only in jurisdictions where they are approved, administered with strict adherence to the manufacturers' instructions, and with careful consideration for the safety of non-target flocks.

Currently available MG vaccines have shown little potential for use in turkeys (6, 73, 218, 240). The F strain vaccine is too pathogenic (243, 253), and the ts-11 vaccine appears to have little or no ability to colonize turkeys (6, 218, 396, 399). A vaccination trial in turkeys using 6/85 vaccine resulted in little or no protection against airsacculitis after heavy aerosol challenge, but there was some protection detected against development of lesions in the upper respiratory tract (218).

Efforts continue to development new and improved MG vaccines. A modified live MG vaccine (strain GT5) was constructed by reconstitution of the avirulent high passage R strain (R_{high}) with the gene encoding the major cytadhesin GapA (179, 320). A naturally low virulent MG isolate (K5054) from turkeys, genotypically similar to the house finch strain, has shown potential for

use as a vaccine in chickens and turkeys (122, 123). A recombinant fowlpox-MG vaccine has been introduced (44).

Treatment

M. gallisepticum has shown sensitivity *in vitro* and *in vivo* to several antimicrobics including macrolides, tetracyclines, fluoroquinolones, and others but is resistant to penicillins or other antibiotics which act by inhibiting cell wall biosynthesis (50, 183, 188, 203, 236, 250, 360, 381, 391). *M. gallisepticum* may develop resistance, and demonstrate cross-resistance, to commonly used antibiotics (50, 142, 285, 332, 403, 425). Techniques for *in vitro* antimicrobial susceptibility and minimum inhibitory concentration (MIC) testing have been described (50, 160, 376). Antimicrobics have been used to treat MG respiratory diseases (149, 183, 184), and to reduce egg production losses (315) and transmission (301, 314, 348, 360, 415). Antimicrobics may reduce the severity of clinical signs and lesions, and significantly reduce populations of MG in the respiratory tract (84, 189).

Attempts to treat CRD with various antimicrobics during the 1960s produced variable results. In many cases, it was doubtful if small increases in weight gain or egg production, and moderate reduction of carcass condemnations, were sufficient to cover medication costs. However, some more commonly employed treatments that tended to provide favorable results included use of oxytetracycline or chlortetracycline at 200 g/ton feed for at least several days. Tylosin has been injected subcutaneously at 3–5 mg/lb body weight or administered at 2–3 g/gal drinking water for 3–5 days. Administration of very low levels of tylosin in feed to MG-exposed layers in multiple-age complexes was found to lessen egg production losses (315). Tiamulin and tiamulin plus salinomycin were reported to be effective treatments in chickens or turkeys (15, 35, 361). Attempts to eliminate egg transmission of MG by medication of breeder flocks or their progeny with streptomycin, dihydrostreptomycin, oxytetracycline, chlortetracycline, erythromycin, or tylosin generally were able to produce considerable reduction in rates of MG infection but generally were not adequate to obtain entirely infection-free flocks. Tylosin and gentamicin injection of chicken breeder hen eggs, and spectinomycin and lincomycin treatment of chicks showed efficacy (301). Efficacy has been shown with: spiramycin in layer chickens (15); fluoroquinolones in broilers (25, 184, 186, 199, 202, 285, 375), breeder chickens (360), and layers (314); and tilmicosin in chickens (71, 185, 201, 351) and turkeys (187).

Regulations on the use of antimicrobials in poultry are rapidly evolving, vary considerably among jurisdictions (e.g., European Union, United States, etc.) and should be consulted just prior to treatment for verification and currency. In the United States, see the AVMA Judicious Therapeutic Use of Antimicrobials in Poultry, American Association of Avian Pathologists Guidelines to Judicious Therapeutic Use of Antimicrobials in Poultry (10); AVMA Judicious Use of Antimicrobials for Poultry Veterinarians (13); Animal Medicinal Drug Use Clarification Act (11); and the FDA Approved Animal Drug List (Green Book) (12).

Egg injection or dipping with a temperature or pressure differential has been used to introduce antimicrobials into hatching eggs to control MG *in ovo* transmission (8, 145, 159, 301, 312, 363). In general, these methods greatly reduced, but sometimes did not completely eliminate, the possibility of egg transmission. Effects on hatchability were not consistently favorable, and bacterial contamination was troublesome at times. However, the use of antimicrobials for egg injection or dipping made it possible to obtain sufficient MG-free birds to provide the poultry industry with a nucleus for producing clean progeny for large flocks, resulting in MG-free chicken and turkey breeder flocks in the United Sates. An alternative approach for reducing *in ovo* transmission of MG involved heating eggs in a forced-air incubator during a 12–14-hour period to reach an internal temperature of 46.1°C (411). Hatchability was sometimes reduced (2–3% to 8–12%), but field studies demonstrated adequate success in many cases (154, 284, 348). Complete elimination of MG from all birds in an infected flock by mass antimicrobial therapy is an unrealistic expectation, and treatment should be regarded as a method for short-term amelioration of disease and economic effects, rather than as a long-term solution to the problem (240, 331, 395).

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Mycoplasma meleagridis Infection

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Introduction

Definition and Synonyms

Mycoplasma meleagridis (MM) (N strain PPLO, H serotype) is a specific pathogen of turkeys. It is the cause of an egg-transmitted disease in which the primary lesion is an airsacculitis in the progeny. Other manifestations include decreased hatchability, skeletal abnormalities, and poor growth performance.

Economic Significance

Economic losses caused by MM in turkeys have been associated primarily with egg-borne infections. During the early 1980s when the prevalence of MM was very high, the monetary cost to the U.S. turkey industry resulting from MM-related hatchability losses and the cost of egg treatment to control egg-borne infections was estimated at 9.4 million dollars per year (26). Currently, the economic losses due to MM infection in turkeys have been reduced significantly with the availability of MM-free eggs and poults supplied by major turkey breeders.

Public Health Significance

M. meleagridis infections in turkeys have no public health significance.

History

In 1958, Adler *et al.* (4) were the first investigators to show that airsacculitis in poults hatched from infected eggs could be associated with a mycoplasma other than *M. gallisepticum*. The mycoplasma, later named *M. meleagridis*, was isolated from the air sac lesions of poults originating from 8 breeding flocks from 4 states. The clinical syndrome of airsacculitis and/or associated skeletal abnormalities has been called day-old type airsacculitis (81), airsacculitis deficiency syndrome (113), and turkey syndrome-65 (TS-65) (143).

Etiology

Classification

M. meleagridis (153) was designated as the N strain by Adler *et al.* (4) and placed in the H serotype by Kleckner (73), Yoder and Hofstad (169), and Dierks *et al.* (35).

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Morphology and Staining

Giemsa-stained smears of broth cultures of MM show coccoid bodies approximately 0.4 μm in diameter, similar to those of *M. gallisepticum* (153, 169). They appear singly, in pairs, or in small clusters. Ultrastructure studies (140) showed that MM did not possess bleb structures typical of *M. gallisepticum*, but had thicker fibrils in the central nuclear area. In both species, ribosomes were distributed in uniform rings around the cell peripheries. Similar studies by others (60) revealed that the predominant morphotype of MM was a spherical form ranging from 200–700 nm in diameter. Other forms (chains of *Streptococcus*-like cells) suggested replication by binary fission. Similar forms, including short filaments, have been observed by scanning electron microscopy (68). An acidic mucopolysaccharide capsule was demonstrated. The DNA of type strain 17529 has a guanine and cytosine (GC) base composition of 27.0–28.1% and a genome size of $4.2 \pm 0.5 \times 10^8$ daltons, both figures being at the lower range exhibited by mycoplasma (5).

Growth Requirements

M. meleagridis is a facultative anaerobe. Growth is optimal at 37–38°C and slight at 40–42°C. Most isolates do not adapt readily to broth media (44, 148). Serum or serum fraction (Difco) is an essential ingredient for growth. Swine and horse sera are satisfactory, but chicken and turkey sera are not (148).

A number of media have been described for cultivation of MM (148). A satisfactory broth consists of mycoplasma broth powder (2.1%), yeast autolysate (1%), and heat-inactivated (56°C for 30 minutes) horse serum (15%) (98, 118, 153). For solid medium, Bacto agar (1.2%) is added to the formulation. The pH of the final medium is 7.5–7.8. Fresh yeast extract (48) may be substituted for the dehydrated product. Another commonly used medium is modified Frey's medium (49) described under "*Mycoplasma synoviae* Infection". A broth medium designated SP-4, containing cell culture medium components, also supports excellent growth of MM (44).

The fastidious nature of this organism is exemplified in the observation that from time to time, certain batches of media do not support growth of the organism. In such cases, the source of the problem often can be traced to any one of the ingredients, including the water, used in the medium.

Colonial Morphology

Colonies on agar medium after 2–3 days incubation appear small and flat (0.04–0.2 mm in diameter), with rough-appearing

centers of ill-defined nipples. Nippling of the colonies is more prominent in laboratory-adapted strains than in fresh isolates (153).

Biochemical Properties

The organism does not ferment dextrose or other carbohydrates or reduce tetrazolium salts (153, 169) but uses arginine (67), and has phosphatase activity (70). Horse erythrocytes incorporated into turkey meat infusion agar are hemolyzed by MM (169).

Susceptibility to Chemical and Physical Agents

Very little is known about susceptibility of MM to chemical and physical agents. It is assumed, however, that most chemical disinfectants would be effective against it (23).

In broth at pH 8.4–8.7, MM may survive up to 25–30 days at high titers, 10^7 colony-forming units (CFU)/ml (34). Freshly seeded cultures on agar will survive for at least 6 days at room temperature (72, 153). The organism survives for at least 6 hours in the air (9). *In vitro* inactivation of four strains of MM at 45°C varied from 6–24 hours, although at 47°C inactivation of two strains occurred between 40–120 minutes (89).

Isolates of MM may be maintained for at least 2 months by mincing colonies on agar in 3% sucrose and freezing at –20 to –70°C. Yoder and Hofstad (169) found broth-overlaid agar slant cultures to be viable after at least 2 years storage at –30°C. Lyophilized cultures remain viable indefinitely (149). The organism does not decline in substantial numbers in turkey semen during cryopreservation and subsequent thawing (46).

Antigenic Structure

M. meleagridis is antigenically unrelated to all other avian mycoplasmas. Use of hyperimmune polyclonal rabbit antiserum as well as monoclonal antibodies raised against MM shows antigen heterogeneity among strains. Moreover, analysis of epitopes with monoclonal antibodies reveals that, as for other mycoplasma species, some epitopes are not expressed in all strains (2, 36). The agglutination (4, 169), fluorescent antibody (FA) (31), antiglobulin (3), growth and metabolic inhibition (35, 44, 90, 108), and complement fixation (50, 106) tests have been used to identify MM.

A few isolates possess hemagglutination activity (119, 139, 153). When hemagglutinating and nonhemagglutinating strains of MM were compared by polyacrylamide gel electrophoresis and simple and two-dimensional immunoelectrophoresis, minor antigenic differences were observed in the latter test only (44). Rhoades (123) showed that the determinant group(s) responsible for hemagglutination differed from that of agglutination.

The organism possesses a heat-stable lipid or polysaccharide toxin that causes an increase in ceruloplasmin activity when injected intravenously into chickens (33). The relationship of this toxin to the capsular material described by Green and Hanson (60) and to hemagglutinating activity is not known. However, hemagglutinating activity is not an essential component for virulence, because strains lacking this activity may be highly pathogenic (153, 164).

Strain Classification

Pathogenic and nonpathogenic strains of MM were described by Ghazikhanian and Yamamoto (54, 55). Of three strains studied, one failed to multiply *in vivo*, another multiplied but failed to produce lesions, although the third multiplied and produced airsacculitis. Zhao *et al.* (172) showed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) that these strains differed in their cell protein profiles. Strain variations may account for the variability in clinical manifestations attributed to this organism (42).

Experimental infection of turkey embryos and tracheal explants with MM results in deciliation and sloughing of epithelial cells (83). When inoculated into the allantoic cavity of 17-day-old turkey embryos, MM induced curved toes and histological studies including scanning electron microscopy, immunohistochemistry, laser capture microdissection and PCR revealed fissures in the cartilage, cellular infiltration within the bones and the presence of MM in tarsometatarsal affected joints (85). MM was also found to cause alterations to the cellular surface topology and necrosis on the surface of the eggshell membranes (84).

Pathobiology and Epidemiology

Incidence and Distribution

Early studies showed that MM was a common pathogen of turkeys with a worldwide distribution (4, 10, 61, 91, 116, 127, 135, 142, 143). These prevalence studies, together with the knowledge that MM was transmitted through the egg, led major primary breeders in the mid-1970s to initiate programs to eradicate the agent from their stocks (60). With the success of these programs, the prevalence of MM has been reduced significantly within the past 20 years in the major turkey-producing areas in the world (see "Eradication").

Natural and Experimental Hosts

M. meleagridis is a specific pathogen of turkeys. When injected into turkey embryos by the yolk sac route, the organism produces a high incidence of airsacculitis but causes minimal mortality (150). The high infectivity and low mortality caused by MM in turkey embryos under experimental and natural conditions indicates that it has attained an ideal host-parasite relationship.

When inoculated into the yolk sac of chicken embryos, MM multiplies to high titers without causing high mortality (158, 169), but abnormally shaped toes and severely denuded tracheae may be observed (82). Turkeys of all ages are susceptible to air sac infection with MM when inoculated via the air sac or trachea (79, 99, 152). Chickens are refractory to infection with MM (2, 151). MM was reported to have been isolated from free-ranging birds of prey in Germany (88) and found to infect peafowl, pigeons and quails (66). Antibodies were also detected in lesser prairie-chickens (62).

Transmission

Vertical Transmission

M. meleagridis is perpetuated primarily through egg transmission. Infection of the female reproductive tract occurs as an endogenous infection during embryonic development (93), as an

ascending infection from foci in the cloaca or bursa of Fabricius after the occluding plate is perforated at sexual maturity (92), or by insemination of hens with MM-containing semen (79, 98, 100, 155, 161). Infection rates of 19–57% have been found in flocks in which cultures were taken from the vagina of virgin females. Although such hens contribute to the overall egg-transmission rate, particularly when the incidence is high, insemination with mycoplasma-contaminated semen plays a major role in sustaining the egg-transmission rate during the laying season (75, 79, 162). The egg-transmission rate among individual hens may vary from 10–60% (155). Apparently, however, no regular pattern exists as to the sequence of infected eggs laid (98). Transmission starts out at a low rate during the first 2–3 weeks of lay, reaches a maximum at midseason, and gradually declines toward the end of the laying season (14, 79). There seems to be some intracyclic fluctuation in the transmission pattern during the laying season (79, 162), but it has not been possible to relate such changes to the insemination schedule.

Egg transmission does not occur in hens in which the organism is found only in the upper respiratory tract (sinus) (79, 98, 147) and is minimal in hens infected via air sac and subsequently inseminated with clean semen (79).

A comparative study of persistence of MM, *M. synoviae*, and *M. gallisepticum* in the genitalia of adult turkeys indicated that MM favored this environment more than the others (147).

Although the exact site in the reproductive system where the organism infects the developing egg is not known, it appears not to be in the ovary; several studies have generally failed to yield the organism from the ova of hens known to be transmitting the organism through their eggs (98, 147, 155). Furthermore, egg transmission occurs at a high rate in the absence of active abdominal airsacculitis.

The organism has been recovered from various sites of the oviduct, with the greatest frequency from the vagina and uterus (100, 155). In hens repeatedly inseminated with MM-contaminated semen, high levels of infection in the uterovaginal region were not sustained, although such hens did transmit the organism through their eggs (149). In hens inseminated with contaminated semen, the organism was found as high as the magnum (79). The organism has been isolated from the shell membrane and vitelline membrane-yolk of preincubated eggs from naturally infected turkeys, but at higher rates from the latter (10–12%) than former (2–4%) sites (57). Mycoplasma counts of 10^3 to 10^5 CFU/vitelline membrane have been obtained (149). Thus, although the organism has the potential to infect the developing egg at various sites in the oviduct, the critical site appears to be in the area of the fimbria or magnum.

As is the case with the female, cloacal infection detected in the male at the time of hatch can persist through sexual maturity; semen taken from such males will contain the organism (147, 167). The organism remains localized in the cloaca and phallus and does not ascend the vas deferens or testes (118, 147). Isolation rates of MM from the phallus or semen of naturally infected male flocks have ranged from 13–32%. Histologic study of the phallus and accessory organs suggests that a possible site of localization is the region of the submucosal gland (52).

Horizontal Transmission

Direct and indirect transmission of MM may occur at any stage of the bird's life. Direct transmission by the airborne route may occur within a hatchery (79) or flock (160), or on occasion between flocks separated by 1/4 mile (57). Airborne transmission in mature turkeys usually results in a high infection rate (up to 100%), which remains localized in the sinus and trachea (79, 98). In young birds during the brooding and growing periods, however, the organism may localize in the genitalia of approximately 5% of the birds infected by the respiratory route (160).

Indirect transmission results from management practices including sexing, vaginal palpation, artificial insemination, and vaccination whereby mycoplasmas are carried manually from infected to noninfected turkeys via contaminated hands, clothing, and equipment (57, 98).

Airborne transmission apparently is of little significance after a bird has reached sexual maturity. Thus, egg transmission does not occur in noninfected females that have been placed in cages adjacent to infected females. Similarly, clean males held in the same room with phallus-infected males produce MM-free semen throughout the production period (147, 149).

Clinical Signs

Despite a high rate of airsacculitis in poults originating from infected dams, respiratory signs are rarely observed. Lateral transmission that may occur by direct or indirect means in adult birds may lead to a high infection rate, but rarely to clinical disease. Thus, *M. meleagridis* commonly occurs as a silent infection in adult birds.

Although not a consistent feature of the disease, the syndrome called TS-65 (also called airsacculitis deficiency syndrome) may be associated with MM egg-borne infection (59). The syndrome, which includes signs of bowing, twisting, and shortening of the tarsometatarsal bone and hock joint swelling, has been reproduced experimentally in MM-free poults (15, 105, 145, 146, 156). Deformation of cervical vertebrae (24, 101), stunting, and abnormal feathering (15) are additional features of the disease.

M. meleagridis acts synergistically in producing severe airsacculitis with *M. iowae* (126) and sinusitis with *M. synoviae* (117). In a flock naturally infected with MM and *M. synoviae*, sinusitis was estimated to be 2.1% in males and 0.13% in females (117). Although it is generally believed that neither agent alone is capable of producing sinusitis, field cases have been encountered in which only MM has been isolated from sinus exudate.

Morbidity and Mortality

Reproductive Performance. M. meleagridis does not adversely affect egg production or fertility and does not cause early incubation mortality (30, 159). It causes late incubation (25–28 days) mortality in artificially (26, 159) and naturally (38) infected turkey embryos. It has been estimated that MM causes a loss in hatchability of 5–6% of fertile eggs set under commercial conditions (41). Edson (38), using risk analysis, determined the mortality rates of embryos naturally infected with MM and/or an unidentified mycoplasma. The analysis showed that embryos infected with MM, unidentified mycoplasma, and both agents were

5, 7, and 25 times more likely to die than the mycoplasma-free embryos. The unidentified mycoplasma was later identified as *M. iowae* (149), a common mycoplasma of turkeys known to reduce hatchability (125).

Air Sac Lesions and Condemnations. During the mid-1960s, MM-associated airsacculitis was reported to be one of the major causes of condemnation of fryer-roaster turkeys in the United States (6, 78). Air sac lesion rates of 10–25% in first-run poults from MM-infected flocks over a season's production were reported under experimental and commercial conditions (47, 79, 98, 155).

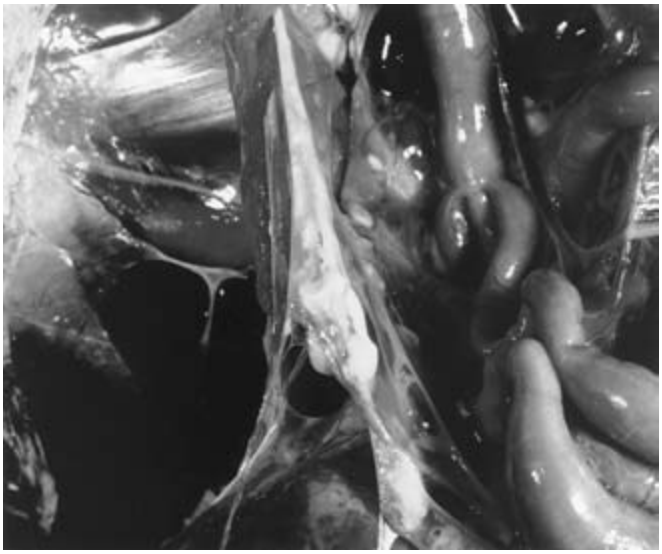
Because air sac lesions caused by uncomplicated MM infection regress within 15–16 weeks (10, 167), it appears that other agents or factors may be involved in the overall picture. Anderson *et al.* (6) observed a twofold or greater increase in incidence of air sac lesions caused by MM in turkeys raised to 12 weeks of age in a high-dust environment.

Brown and Nestor (22) suggested that turkeys selected for low plasma ACTH following cold stress were more resistant to MM infection than those selected for high ACTH levels. Saif *et al.* (131) reproduced complicated airsacculitis with MM and *Escherichia coli* in poults. Mixed infections of MM and *M. iowae* also accentuate the severity of air sac lesions (126). Therefore, a number of interacting factors may aggravate MM-induced air sac lesions in young turkeys.

Skeletal Abnormalities and Growth Performance. In affected flocks, MM-associated skeletal abnormalities (i.e., TS-65 syndrome) may be observed in poults between 1–6 weeks of age (143). Five to 10% of the poults may show clinical signs, but on occasion the percentage may reach higher levels. Not all cases progress to an irreversible state (143). Incidence of the disease seems to increase with progression of the laying season. Mortality is due primarily to cannibalism of affected birds. The problem is not associated with a particular strain of bird, but the male seems to be more susceptible.

Peterson (112) found a positive association of skeletal lesions, airsacculitis, and high agglutination titers to MM in poults with the TS-65 syndrome, which supports the view that the syndrome is initiated by a generalized egg-borne infection (146). It was further hypothesized, based on *in vitro* and *in vivo* studies, that the organism may deprive the embryo of biotin, resulting in abnormal bone development (15, 17). Others have postulated that the organism may compete for arginine, an essential amino acid for proper bone development (156). *In vitro* studies indicated, however, that MM strains of varying virulence did not differ in their arginine requirement; also, significant differences in plasma arginine concentration between noninfected and infected poults with leg abnormalities were not observed (67).

Nelson *et al.* (105) distributed MM-free and MM-infected eggs in large numbers to 11 cooperators in 8 states to study the leg weakness syndrome under commercial conditions. The results indicated that the total daily mortality, number of cull poults, and skeletal deformity were much lower in poults hatched from MM-free eggs. A significant advantage in weight gain was also observed with MM-free vs. infected poults (13, 107, 143).



21.5. Airsacculitis in a 4-week-old turkey caused by eggborne *Mycoplasma meleagridis* infection.

Conversely, others (25, 28, 29) were unable to demonstrate any economic advantage of MM-free over MM-infected turkeys. Possible reasons for the divergent results are not clear, but factors such as differences in the genetic makeup of the bird, virulence of MM strains, environmental stresses, and secondary infections may influence the picture.

Pathology

Gross

Although gross lesions, if any, in poults at time of hatch from infected dams are limited to the air sacs, the organism may be widely distributed in various tissues including feathers, skin, sinus, trachea, lungs, air sacs, bursa of Fabricius, intestine, cloaca (12, 115, 121), and hock joints (149). The air sac lesions are characterized by thickening of the air sac walls with adherence of a yellow exudate to the tissue and, occasionally, presence of variously sized flecks of caseous material free in the lumen (4). Extension of such lesions to the abdominal air sacs is a common occurrence by 3–4 weeks of age. It is also possible for the organism to be present in air sacs of day-old poults exhibiting no lesions; in such cases, air sac lesions may develop in 3–5 weeks (10). Lesions produced by MM when not mixed with *M. iowae* are not as extensive or fulminating as those described for *M. gallisepticum* (35, 81). Figure 21.5 shows a poult, hatched from an MM-infected egg, with caseopurulent airsacculitis.

Skeletal lesions, when present, usually are associated with severe airsacculitis (112). Sternal bursitis (152), synovitis (131), and ascites (145) are additional lesions observed in experimental infections. The sinusitis produced by MM and *M. synoviae* in mixed infections contains clear mucous to caseous exudate (117). Leg abnormalities in poults hatched from MM-infected eggs are shown in Figure 21.6.



21.6. Bowing of tarsometatarsal bones of a 3-week-old turkey hatched from a *Mycoplasma meleagridis*-infected egg.

Microscopic

In embryonic infection with MM, exudative airsacculitis and pneumonia were the only inflammatory lesions seen. Lesions that developed at 25–28 days of age were related to maturation of inflammatory cells. Air sac lesions consisted predominantly of heterophils with some mononuclear cells, including lymphocytes and varying amounts of fibrin and cellular debris. Epithelial necrosis was seen in severely affected air sacs. Mononuclear cells and fibrin were the prominent features of lung lesions (55, 121). Significant or marked microscopic changes in other organs in embryos or poults were not observed despite invasion of the organism into many of these sites (55).

In 7-week-old poults infected with MM by the air sac route, lymphocytic perivascular infiltration and fibrinoheterophilic exudate were observed in 2 days. Some areas of the air sac epithelium became hyperplastic and others underwent necrosis at about 4–8 days. Lymphoid follicles were observed in 16 days. When examined by electron microscopy, the follicles were found to be surrounded by encapsulated collagen bundles and composed of hemocytoblasts of bursal origin presumed to be involved in antibody formation (120). Others observed similar sequential changes in poults infected as embryos or at 1–3 days of age (7, 55, 102).

Wise *et al.* (143) indicated that gross and microscopic long-bone lesions of TS-65 were similar to those observed in perosis of dietetic origin. The main lesions were seen in proximal ends of the long bones. Cartilage farthest from blood vessels descending into the proliferative zone from the cartilaginous epiphysis lacked cell density and contained abnormal-appearing chondrocytes. In long-standing cases of 6–8 weeks or longer, growth plates often were normal, suggestive of repair, even though the bones were grossly deformed. These cellular changes in the proliferative zone of the growth plates were seen in all long bones examined, suggestive of a generalized response. It was postulated that MM causes a secondary block of nutrients to the growth plates.

A secondary lesion in the medial side of the proximal end of the tarsometatarsal bone of chronic cases with varus deformity was described as a dyschondroplasia or chondrodystrophy resulting from partial failure of the metaphyseal blood supply at the growth plates (143).

Mild mononuclear cell infiltration was observed in the periarticular region of the hock joint in 2-week-old poults inoculated with MM intravenously (114).

The most prominent lesion in hens infected by the vaginal route was focal encapsulated accumulation of lymphocytes present most frequently in the fimbria, uterus, and vagina. Plasma cells and heterophils were also present in significant numbers in the lamina propria of the reproductive tract. The encapsulated follicles were believed to be active in antibody formation (122). Similar lesions in the reproductive tract of turkeys infected with MM were described by Ball *et al.* (8).

Gerlach *et al.* (52) examined histologically the phallus and accessory structures of males experimentally infected with MM. The only significant change was an extensive lymphofollicular formation in the region of the mucous-type glands in the submucosa of the lymphfold.

Immunity

Active

Turkeys inoculated intravenously or by the respiratory route with MM were resistant to reinfection when challenged by the same routes 21 weeks later. There was no correlation, however, between antibody titer and resistance (99). Repeated injections of 20-week-old hens with live organisms failed to induce protective immunity or reduce egg transmission (141).

When hens that had been vaginally infected artificially with MM culture or with contaminated semen were subsequently inseminated with clean semen, the organism was eliminated from the vagina within 4–14 weeks, although hens continuously inseminated with contaminated semen maintained a high incidence of infection (75, 79). However, insemination with clean semen of virgin hens known to be vaginal carriers of MM resulted in a high egg-transmission rate and persistence of oviduct infection (38, 162). In the first-mentioned studies, it appears that an active immune mechanism was functioning to eliminate the organism after removal of the source of infection, i.e., contaminated semen. Persistence of infection in the latter study may be an expression of immune tolerance in hens infected by egg transmission.

Yamamoto *et al.* (166) found that hens infected with MM via the oviduct during one breeding season were free of MM at the start of the second laying season; among five adult males infected via the phallus, the organism persisted for 55–344 days. These findings are consistent with the observation that the decline in the egg-transmission rate during the latter part of the laying season may be related to an active immune response. A study by Ortiz *et al.* (109) suggested that MM infection of the bursa of Fabricius during embryonic development causes an impairment of the secondary antibody response to innate or inactivated antigens.

Passive

Maternal antibodies (agglutinins) may be detected in a high percentage of poults from infected dams, and persist for approximately 2 weeks post-hatching. Such antibodies are not protective against the development of air sac lesions in infected embryos (99, 155). Conversely, purified IgM and IgG antibodies when injected into the yolk sac of infected embryos significantly reduced embryo mortality and the incidence of leg deformities in hatched poults, but they did not reduce air sac lesions or isolation rates when compared with the controls (18).

Diagnosis

Isolation and Identification of Causative Agent

Bacterial Isolation and Identification

M. meleagridis may be isolated readily on several commercially available and laboratory-prepared media (see "Growth Requirements"). Thallium acetate (1:4000) and penicillin (1000 units/ml) are inhibitors added to agar plates, slants, and broth. Polymyxin B (100 units/ml) may be added to the broth portion of the overlay to facilitate isolation of MM from highly contaminated sources such as the cloaca and phallus. Mycostatin (50 units/ml) may be added to the agar and broth to inhibit fungi (100). *M. meleagridis* may be selectively isolated from specimens containing mixed cultures by adding to the medium immune serum against the undesired mycoplasma (20). The organism may be isolated from vitelline membrane, air sacs, intestine, and many other sites of infected embryos (see "Pathology, Gross"). It may also be isolated from the kidneys of poults infected by the air sac route (145).

For large sampling studies in the field (e.g., cultures from the trachea, palatine cleft, vagina, or phallus), placing swab specimens in overlay broth (4) facilitates transport to the laboratory; the broth also serves as an initial enrichment (110). At necropsy, the organism may be isolated from various sites of the respiratory (including sinus) and reproductive systems.

When growth is apparent in the original isolation medium, usually after 4–6 days of incubation, agar plates are streaked and placed in a sealed container with added moisture. Plates are incubated at 37°C for 5–7 days before being examined for colonies under the dissecting microscope, and are incubated at least 10 days before being discarded as negative.

M. meleagridis may be differentiated from other chicken and turkey mycoplasmas by its inability to utilize glucose and its ability to metabolize arginine and phosphate (67, 70, 135, 169). Definitive identification, however, must be based on serologic methods. The direct (31) and indirect (20) FA, growth inhibition (35), and immunoperoxidase (69, 134) tests are commonly used for this purpose. In addition, an antigen-capture ELISA has been developed for detection of mycoplasma antigen directly in broth culture (1).

Antigen Detection

DNA-based tests have recently been developed for the direct detection of the organism in clinical specimens (19, 45, 51, 86, 96, 97, 170, 171). Main advantages of PCR-based methods are their rapidity and their ability to detect MM within a high background flora as in the case of cloacal swabs or from samples collected on antibiotic-treated birds.

Serology

The rapid plate (RP) and tube agglutination (TA) tests are effective in detecting MM infections. Antibodies are detected in poults hatched from infected eggs in 3 weeks and in turkeys infected by contact in 4–5 weeks. Birds with active air sac lesions may have high agglutinin titers, and those with localized infections in the sinus or phallus may be negative or show low titers (2, 99, 167). The RP test may be quantified by performing it on serially diluted serums. A reaction at a dilution of 1:5 is significant (164, 167), but for flock diagnosis, some samples should react at 1:10 or higher. Each lot of antigen should be pretested with a standard positive serum for its reactive quality at higher serum dilutions.

The hemagglutination-inhibition (HI) test (119, 139) is another useful test for detecting antibodies to MM infections. Although nonhemagglutinating laboratory strains of MM do not elicit high HI antibody responses in turkeys, the HI test is very effective in detecting such antibodies in naturally infected birds (123). Apparently, field infections with MM occur with strains possessing hemagglutinating activity, but this characteristic is quickly lost for most strains when they are cultivated on laboratory media (123). Furthermore, because the antigenic determinant(s) responsible for hemagglutination differs from that of agglutination (123), it is possible to find individual turkeys in an infected flock whose serum will be positive in the HI and negative in the TA tests; the reverse situation may also occur (164).

Yamamoto *et al.* (164) adapted the HI test to the microtest system. Using four units of antigen, titers of 1:40 were considered suspects and 1:80 or greater as reactors. When used as a confirmatory test to the RP test, a positive HI signifies infection, although a negative HI requires a more conservative interpretation and may involve use of other confirmatory tests or follow-up testing for final diagnosis (165). The micro-HI test has been used to identify false-positive RP reactions (149) in flocks recently vaccinated with *Erysipelothrix* vaccine (16).

Kleven and Pomeroy (74) found that the RP test detected IgM; the TA test detected both IgM and IgG; and the HI test detected IgG most efficiently. However, the early HI antibody response of turkeys to high doses of MM given intravenously was of the IgM class (124).

Other tests developed for mass screening are the microagglutination (157), indirect or blocking enzyme-linked immunosorbent assay (ELISA) (37, 111), and avidin-biotin enhanced dot-immunobinding assay (32).

Differential Diagnosis

Air sac lesions caused by MM must be differentiated from those caused by *M. gallisepticum*, other Mycoplasma serotypes, and possibly other agents. The possibility of a mixed infection of MM with *M. synoviae* or *M. iowae* should be considered if embryo mortality, sinusitis, or airsacculitis is observed. *M. meleagridis*-associated skeletal abnormalities must be differentiated from similar lesions caused by *M. iowae* or of dietetic origin.

Intervention Strategies

Management Procedures

Although early studies placed much emphasis on the control of MM infections in turkeys by use of various antibiotic treatment regimens (see "Treatment"), the goal of primary breeder organizations was to eradicate the agent from their stocks. Because virtually all breeding stocks were infected, a program of test and slaughter—which had been so effective in the control of *M. gallisepticum*—was not a practical approach for eradicating MM (163). Experimental studies demonstrated that the administration of antibiotics into eggs either by dipping or by inoculation into the air cell (65) or the small end (43, 94, 95) were useful methods to reduce the egg-transmission rate. Heat treatment of eggs (168) was not effective in eliminating MM from turkey eggs (61, 71, 143). Tylosin (76) or gentamicin (129) was not effective, but spectinomycin (0.6 mg/ml of diluent) (128) was effective in eliminating MM from turkey semen. These studies laid the foundation for effective eradication programs that followed.

Eradication

The basic principles and procedures for producing MM-free breeders include: 1) reduction of genital infection to minimal levels by serology and culture. The egg-transmission rate can be reduced significantly by using males that are not genital carriers. Males that yield three consecutive negative cultures from the phallus or semen are usually free of infection. Eliminating vaginal carriers can reduce the infection rate even further. Even a single sampling will identify most carriers. Cultures from males are taken a few weeks before their use as breeders, and those from females are taken from the cloaca before or from the vagina during egg production. Special care must be taken to prevent cross-contamination when specimens are taken for culture or during insemination (39). 2) Treatment of eggs with an effective antibiotic(s) by dipping and/or injection. Because these procedures may reduce hatchability by 10% or more, pretrials should be conducted before embarking on a large program. The possibility of developing antibiotic resistant strains of MM should be kept in mind, particularly at the primary breeder level where treated birds must be recycled in the operation (53, 95, 146). 3) Hatching of eggs in MM-free hatchers and isolation rearing of the turkeys. Because MM can be introduced onto a farm in a number of ways (see "Transmission") and usually occurs as an unapparent infection, it is essential that a high level of biosecurity be maintained. 4) Serologic and cultural monitoring of the treated flock at 16 weeks of age and periodic intervals thereafter, and elimination of infected groups.

Using the principles just outlined, MM-free turkeys have been produced experimentally (147) and commercially (56, 57, 80, 107, 144). A treatment regimen that was used to eradicate MM from a primary breeder organization consisted of dipping eggs in a solution of gentamicin sulfate (750–900 ppm) followed by injection with a solution containing 0.6 mg gentamicin and 2.4 mg tylosin/dose into the small end of the egg (57).

Edson *et al.* (40) developed an equation based on the Poisson distribution to predict the chance of success of eradicating MM: $p(0) = e^{-na\beta h}$, where the probability of success p(0) was described by n, the number of eggs treated; a, the pretreatment infection rate of the eggs; ß, the treatment failure rate; and h, the hatchability of treated eggs. Decreasing the size of any or all of the four parameters increases the likelihood of eradication. This predictive equation is a useful quantitative tool for management decision-making. Currently, the primary breeder organizations that are the major genetic source for commercial turkeys worldwide are free of MM.

An economic decision analysis was described by Carpenter *et al.* (27) to assist commercial multiplier breeders to determine the economic advantage of eradicating MM.

A program for certifying freedom from MM infection of turkey breeding stocks under the National Poultry Improvement Plan (NPIP) was initiated on January 1, 1983 (133). According to the 2005 NPIP testing summary, 98.3% of 579 multiplier breeder flocks representing 4.5 million breeders qualified as "U.S. MM Clean" (103). These data and an industry-based survey conducted in 1994 suggest that significant progress has been made in reducing the prevalence of MM in the turkey industry because MM-free stock first became available in the early 1980s (57, 58, 77). Similarly, a very low prevalence of MM infections is observed in European breeder and meat flocks. In the European Community, disease surveillance programs provided in the Council Directive 90/539/EEC on health conditions governing intra-Community trade in, and imports from, third world countries of poultry and hatching eggs, include MM in turkeys.

Vaccination

Vaccines are not available for prevention of MM infection in turkeys.

Treatment

Antibiotics having *in vitro* activity against MM include gentamicin (130), tylosin, tetracycline (154), spectinomycin-lincomycin (63), tiamulin, spectinomycin and spiramycin (87), doxycycline and the fluoroquinolones (138), and josamycin (136). In trials conducted with turkey embryos, tylosin was the most active; tetracycline, chlortetracycline, and streptomycin were variable; and erythromycin showed no activity against two isolates of MM (154).

A combination of lincomycin and spectinomycin administered at 2 g/gal of water for 5 days (64) or tiamulin at a concentration of 0.025% in the drinking water for 3 days (137) had therapeutic activity against MM infections. Enrofloxacin was effective in reducing mortality of poults having complicated MM infections (21). Parenteral injections or water medication with tylosin of turkeys in production did not reduce egg transmission (14, 78). Dipping of hatching eggs in antibiotic solution, however, significantly reduced the incidence of air sac infection (11, 78, 112, 132) concomitant with improved hatchability (78, 132), improved performance (11, 104), reduced incidence of skeletal deformities (104, 112), and reduced condemnation at processing (78, 104).

During the late 1960s to early 1980s, before MM-free eggs and poults were available, it was a common practice for multiplier breeders to dip their eggs in antibiotic solution. Tylosin (3000

ppm) or gentamicin (500 ppm) along with a disinfectant such as quaternary ammonium compound (250 ppm) were used in dip solutions. However, repeated hatching egg dipping in antibiotics to reduce the incidence of MM infection could lead to induction of antibiotic-resistant organisms (53). Eradication of MM from major primary breeding companies has reduced the practice of hatching egg dipping in antibiotics by the commercial industry and currently, most major U.S. multiplier breeder companies dip their eggs only when faced with a potential MM outbreak.

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Mycoplasma synoviae Infection

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Introduction

Mycoplasma synoviae (MS) infection most frequently occurs as a subclinical upper respiratory infection. It may cause air sac lesions when combined with Newcastle disease (ND), infectious bronchitis (IB), or both. At other times, MS becomes systemic and results in infectious synovitis, an acute to chronic infectious disease of chickens and turkeys, involving primarily the synovial membranes of joints and tendon sheaths producing an exudative synovitis, tenovaginitis, or bursitis.

History

Infectious synovitis was first described and associated with a mycoplasma by Olson *et al.* (114, 115). A respiratory form of *M. synoviae* infection occurs (117) and air sac infection results with some isolates of MS when combined with ND and IB vaccination (77). See Jordan (64, 66) and Timms (143) for reviews of the MS literature.

Etiology

Classification

Mycoplasma colonies were observed as satellites adjacent to *Micrococcus* colonies by Chalquest and Fabricant (27), who identified the requirement for nicotinamide adenine dinucleotide (NAD). It was designated as serotype S by Dierks *et al.* (33). Olson *et al.* (118) studied several isolates and proposed the name *M. synoviae,* which was subsequently confirmed as a separate species (67).

Identification is based on typical colony and cell morphology, biochemical characteristics, special requirements for growth, and serologic reactions. Immunofluorescence of mycoplasma colonies is the most rapid and reliable method for identification of field isolates. DNA sequence analysis of the 16S rRNA gene has proven to be useful for mycoplasma identification and in phylogenetic studies (153). The complete genome sequence of a strain of *M. synoviae* has been published (150).

Morphology and Staining

In Giemsa-stained preparations, *M. synoviae* cells appear as pleomorphic coccoid bodies approximately 0.2 μm in diameter. Ultrastructural studies of avian synovium reveal MS in endocytotic vesicles. The mycoplasma cells are round or pear shaped with granular ribosomes. They are 300–500 nm in diameter, lack a cell wall, and are bounded by a triple-layered unit membrane (151). An extracellular surface layer has been demonstrated by electron microscopy by ruthenium red and negative staining (1).

Growth Requirements

Nicotinamide adenine dinucleotide is required for growth (27); however, it may be possible to substitute nicotinamide for the more expensive NAD for production of antigens (32). Serum is essential for growth, and swine serum is preferred (28). Growth on agar is accomplished by incubation of plates in a closed container to prevent dehydration of the agar. The optimum temperature is 37°C.

Excellent growth is obtained using a modification of Frey's medium (44) (Table 21.2) or a medium described by Bradbury (20). For agar plates, use 1% of a purified agar such as ionagar #2, Noble agar, or Difco purified agar. All components except cysteine, NAD, serum, and penicillin may be sterilized by auto-

Table 21.2. Modified Frey's medium.

Mycoplasma broth base (BBL)	22.5 g
Glucose	3 g
Swine serum	120 ml
Nicotinamide adenine dinucleotide (NAD)	0.1 g
Cysteine hydrochloride	0.1 g
Phenol red (1%)	2.5 ml
Thallium acetate (10%)*	5 ml
Potassium penicillin G*	1,000,000 units
Distilled H_2O	1000 ml
Adjust pH to 7.8 with 20% NaOH and filter sterilize.	

*For potentially contaminated specimens, an extra 20 ml of 1% thallium acetate and 2,000,000 units of penicillin per liter may be added. Ampicillin (200 mg/l to 1 g/l) may be substituted for penicillin.

claving at 121°C for 15 min. Cool to 50 C and aseptically add the above components, which have been sterilized by filtration and warmed to 50°C. Pour plates to a depth of approximately 5 mm. Phenol red may be eliminated from agar plates.

On primary isolation, tissue antigens, toxins, and antibodies may be present; therefore, a small inoculum, transferred within 24 hr, or making dilutions of the inoculum in broth may improve results. Transfers are made with a pipette using a 10% inoculum. Inoculation of broth medium with a cotton swab from the trachea, choanal cleft, or synovial or air sac lesion is satisfactory. Plain or charcoal cotton swabs are more effective than rayon swabs, and retaining the swab in the broth medium was more effective than discarding the swab (161), but leaving the swab in the growth medium may lead to increased bacterial contamination of cultures. Direct plating onto agar plates may result in colonies at 3–5 days of incubation, but isolation in broth is more sensitive. Broth cultures should be incubated until a color change of the phenol red indicator from red to orange or yellow is noted (usually after 3–7 days); the culture should then be transferred to an agar plate and subcultured into another broth culture. *M. synoviae* is sensitive to low pH; therefore, cultures incubated for more than a few hr after the phenol red indicator has changed to yellow (pH <6.8) may no longer be viable. Plates are observed for the presence of mycoplasma colonies after 3–5 days using a microscope with indirect or low-intensity lighting at a magnification of approximately ×30.

Colony Morphology

Colonies on solid media are best observed with a dissecting microscope at ×30 using indirect lighting; they appear as raised, round, slightly latticed colonies with or without centers. Colonies range from less than 1 to 3 mm in diameter, depending on number of colonies present, suitability of medium, and age of culture. Growth is seen on solid medium in 3–5 days.

Biochemical Properties

Biochemical characteristics of *M. synoviae* have been described (27, 33). *M. synoviae* ferments glucose and maltose with production of acid but not gas in suitably enriched media. It does not ferment lactose, dulcitol, salicin, or trehalose. *M. synoviae* is phosphatase negative and produces film and spots (67). Most isolates are capable of hemagglutinating chicken and turkey erythrocytes. Its ability to reduce tetrazolium salts is very limited.

Susceptibility to Chemical and Physical Agents

Resistance to disinfectants has not been determined but is probably similar to other mycoplasmas. Day-old chicks placed in contaminated chicken houses which had been cleaned and disinfected and maintained empty for 1 wk did not become infected (45). *M. synoviae* is not stable at pH 6.8 or lower. It is sensitive to temperatures above 39°C. It will withstand freezing; however, the titer is reduced. End points have not been reached, but in yolk material *M. synoviae* is viable at least 7 yr at –63°C and after 2 yr at –20°C. Broth cultures maintained frozen at –70°C or lyophilized cultures maintained at 4°C are viable for several years. Survival occurred up to 3 days at room temperature on feathers and up to 12 hr in the nasal cavity of a volunteer, while survival was less than 1 day on most other materials (30). MS was also detected in environmental samples, including feathers, dust, feed, drinking water, and droppings by culture and by PCR (97). A reverse transcriptase PCR detecting 16srDNA was correlated with viable MS organisms in the environment of infected birds. Positive RT-PCR and culturable MS organisms were found in the environment of a depopulated isolator unit for 3–5 days after depopulation of MS-infected chickens (99).

Antigenic Structure

Serum plate agglutination (SPA) (116), tube agglutination (TA) (147), hemagglutination (16, 146), agar gel precipitin (AGP) (134), and enzyme-linked immunosorbent assay (ELISA) (55, 123, 126) antigens have been studied. Studies utilizing Western blots have characterized the major immunogenic membrane antigens of MS (4, 5). A major immunogenic protein, p41, showed promise as an antigen in a dot ELISA, while p53 and p22 did not perform well (4). The molecular size of the major membrane proteins varied among MS strains (5).

Serum from chickens infected with MS occasionally agglutinates *M. gallisepticum* plate antigen (118, 119). Roberts and Olesiuk (133) suggested that the cross-reactions were related to presence of rheumatoid factor and could be stimulated by tissue reactions. Cross-reactions are minimal when the hemagglutination inhibition (HI) or TA test is used. There are also epitopes shared by *M. gallisepticum* and MS (3, 160). Species-specific monoclonal antibodies against MS have been produced (59). Immunoglobulin G Fc receptors have been identified (85).

A cluster of 45–50 kDa surface proteins is immunodominant in strain WVU-1853 (108); they are size and expression variable and fall into two groups, MSPA and MSPB, which are both associated with hemadsorption, which is also variably expressed. MSPA was shown to be a hemagglutinin. These proteins are coded by a single gene, *vlhA* (14, 109), the product of which is then cleaved to form MSPB and MSPA. This gene has a high degree of identity with the *pMGA*1.7 gene of *M. gallisepticum* and hybridizes with other areas of the genome, suggesting that it is

part of a multi-gene family. Variability in expression of *vlhA* is controlled by homologous recombination events with pseudogenes located in other parts of the genome (2, 15, 61, 111). Cultures of the hemagglutinin negative phenotype expressed truncated versions of PMSB and were less pathogenic than hemagglutination positive cultures (105).

Strain Classification

Available information indicates a single serotype of *M. synoviae* (33, 118), and DNA-DNA hybridization techniques show little heterogeneity among MS strains (159, 160). *M. synoviae* strains can be differentiated using restriction endonuclease analysis of DNA (88, 102). A simpler more rapid procedure for differentiation of MS strains is the random amplified polymorphic DNA (RAPD) technique (37, 38), but results may be inconsistent. Pulsed field gel electrophoresis and amplified polymorphic DNA analysis have also been shown to be a valuable tool for discriminating among strains of *M. synoviae* (34, 38, 98). Sequencing of a portion of the *vlhA* gene, either from cultures or tissue samples, has been useful for partial characterization of *M. synoviae* strains (15, 57).

Virulence Factors

Little is known about virulence factors for MS. Differences in virulence could not be explained by potential virulence factors such as hemagglutination and hemadsorption, attachment to cells, or ciliostasis (91). However, the hemagglutination positive phenotype of MS induces infectious synovitis lesions more frequently than does the hemagglutinin negative phenotype (105).

Pathobiology and Epidemiology

Incidence and Distribution

Infectious synovitis was observed primarily in growing birds 4–12 wk of age in broiler-growing regions of the United States during the 1950s and 1960s. Since 1970s the synovitis form has been infrequently observed in chickens in the United States, but the respiratory form has been seen more frequently. Infection without apparent clinical signs is not unusual. *M. synoviae* infection occurs frequently in multi-age commercial layers (100, 122). Infectious synovitis usually appears in turkeys when they are 10–20 wk old. Breeding stock from all major commercial breeds of chickens and turkeys is largely free of infection. *M. synoviae* is worldwide in distribution.

Natural and Experimental Hosts

Chickens and turkeys are the natural hosts of *M. synoviae.* Ducks (10, 144), geese (9), guinea fowl (125), pigeons (8, 128), Japanese quail (8), pheasants (22), and red-legged partridge (127) have been found to be naturally infected. Pheasants and geese (22, 139), ducks (155), and budgerigars (18) are susceptible by artificial inoculation. *M. synoviae* was isolated from house sparrows (*Passer domesticus*) in Spain (127); Kleven and Fletcher (79) found that sparrows could be artificially infected, but were quite resistant. Rabbits, rats, guinea pigs, mice, pigs, and lambs are not susceptible to experimental inoculation (115).

Natural infection in chickens has been observed as early as 1 wk, but acute infection is generally seen when chickens are 4–16 wk old and turkeys are 10–24 wk old. Acute infection occasionally occurs in adult chickens. Chronic infection follows the acute phase and may persist for the life of the flock. The chronic stage may be seen at any age and in some flocks may not be preceded by an acute infection.

Airsacculitis occurs in day-old and older turkeys in MS-infected flocks. Air sac inoculation of mycoplasma-free turkeys results in airsacculitis (48, 131). Inoculation of 18-day-old chicken embryos via the yolk sac resulted in synovitis and airsacculitis in the chicks (19). *M. synoviae* may be isolated from lesions during the acute phase of the disease, but infection of the upper respiratory tract is permanent (77).

Transmission

Lateral transmission occurs readily by direct contact. *M. synoviae* has been demonstrated in the respiratory tract of contact control chickens 1–4 wk following infection of the principals (117). Spread between batteries in the same room occurs. In many respects, the spread appears to be similar to that of *M. gallisepticum* (120) except that it is more rapid. However, slow-spreading infections have been reported (152). Transmission occurs via the respiratory tract, and usually 100% of the birds become infected, although none or only a few develop joint lesions.

Infection has not been thought to occur as a result of environmental contamination after depopulation of infected premises, but in a recent study day-old chicks became infected after placing them in a contaminated environment, but evidence of infection was not seen until 33 to 54 days of age (99). Birds are infected for life and remain carriers.

Vertical transmission occurs in naturally and artificially infected chickens (25); however, many flocks hatched from infected dams remain free of infection. Vertical transmission plays a major role in spread of MS in chickens and turkeys. Thus, all eggs used for live virus vaccine production should be obtained from MS-free flocks. Experimental infection of broiler breeders resulted in MS infection in the trachea of day-old progeny, infertile eggs, and dead-in-shell embryos 6–31 days postinoculation (149). When commercial breeder flocks become infected during egg production, the egg-transmission rate appears to be highest during the first 4–6 wk after infection; transmission thereafter may cease, but infected flocks may shed at any time.

Incubation Period

Infectious synovitis has been seen in 6-day-old chicks, suggesting that the incubation period can be relatively short in birds infected by egg transmission. The incubation period following contact exposure is generally 11–21 days. Antibodies may be detected before clinical disease becomes evident. In birds experimentally infected by inoculation at 3–6 wk of age with joint exudate from infected birds or yolk from infected embryos, the order of susceptibility and incubation period is as follows: foot pad, 2–10 days; intravenous, 7–10 days; intracranial, 7–10 days; intraperitoneal, 7–14 days; intrasinus, 14–20 days; and conjunctival instillation, 20 days. Birds are also susceptible to intramus-

cular inoculation. Intratracheal inoculation results in infection of the trachea and sinus as early as 4 days and readily spreads to contact birds. Air sac lesions are at a maximum 17–21 days after aerosol challenge (77). The incubation period varies with titer and pathogenicity of the inoculum.

Clinical Signs

Chickens

The first observable signs in a flock affected with infectious synovitis are pale comb, lameness, and retarded growth. As the disease progresses, feathers become ruffled and the comb shrinks. In some cases, the comb is bluish red. Swellings usually occur around joints, and breast blisters are common. Hock joints and foot pads are principally involved, but in some birds most joints are affected; however, birds are occasionally found with a generalized infection but not with apparent swelling of the joints. Birds become listless, dehydrated, and emaciated. Although birds are severely affected, many continue to eat and drink if placed near feed and water. A greenish discoloration of droppings, which contain large amounts of uric acid or urates, is frequently seen. Acute signs described above are followed by slow recovery; however, synovitis may persist for the life of the flock. In other instances, the acute phase is absent or not noticed and only a few chronically infected birds are seen in a flock. Chickens infected via the respiratory tract may show slight rales in 4–6 days or may be asymptomatic. Chondrodystrophy was noted in the opposite leg of chickens inoculated via the foot pad. This may have been due to increased weight bearing stress on the leg opposite the affected leg (103).

Recent outbreaks of MS in brown egg layers in the Netherlands were associated with amyloid arthropathy, which was reproduced experimentally (82, 83, 84).

Air sac infection may occur at any age, but is most often observed as a cause of condemnation in broilers (75). Under field conditions, most air sac lesions resulting from *M. synoviae* infection occur in winter. Progeny of MS-infected breeders may have increased air sac condemnations, reduced weight gains, and reduced feed efficiency.

Experimental aerosol inoculation of hens with MS resulted in a detectable drop in egg production in 1 wk postchallenge; by 2 wk production dropped 18%, and by 4 wk production returned to normal (92). Challenge of commercial layers at 10 weeks of age did not result in egg production losses (24). With naturally occurring infection of adults, however, there is ordinarily little or no effect on egg production or egg quality (101, 122), although instances of egg production losses in commercial layers have been observed. However, challenged layer chickens were characterized by transient heterophilia, lymphopenia, monocytosis, eosinopenia, and basopenia (23).

Turkeys

M. synoviae generally causes the same type of signs in turkeys as in chickens. Lameness is the most prominent sign. Warm fluctuating swellings of one or more joints of lame birds are usually found. Occasionally, there is enlargement of the sternal bursa. Severely affected birds lose weight, but many less severely affected make satisfactory weight gains when separated from the flock. In experimentally infected turkeys (115), the first noticeable sign is failure to grow.

Respiratory signs are not usually observed in turkeys, but MS has been isolated from sinus exudates obtained from turkey flocks exhibiting a very low incidence of sinusitis. Rhoades (130) described a synergistic effect of MS and *M. meleagridis* in producing sinusitis in turkeys. Foot pad inoculation of turkeys may result in total cessation of egg production. Challenge with field isolates of MS indicated that current strains are capable of reproducing synovitis in turkeys (69).

Morbidity and Mortality

Chickens. Morbidity in flocks with clinical synovitis varies from 2 to 75%, with 5–15% being most usual. Respiratory involvement is generally asymptomatic, but 90–100% of the birds may be infected. Mortality is usually less than 1%, ranging up to 10%. In experimentally infected chickens, mortality may vary from 0 to 100%, depending on route of inoculation and dose of inoculum.

Turkeys. Morbidity in infected flocks is usually low (1–20%), but mortality from trampling and cannibalism may be significant.

Pathology

Gross

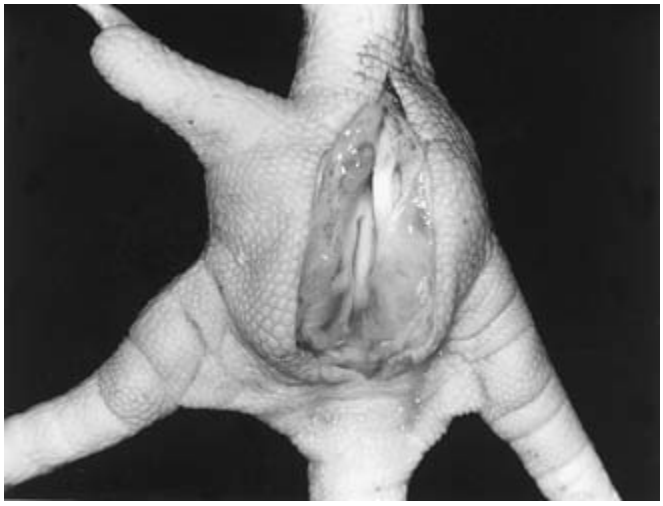
Chickens. In early stages of the infectious synovitis form of the disease, chickens frequently have a viscous creamy to gray exudate involving synovial membranes of the tendon sheaths, joints, and keel bursa, and hepatosplenomegaly (Fig. 21.7). Kidneys are usually swollen, mottled, and pale. As the disease progresses caseous exudate may be found involving tendon sheaths, joints, and extending into muscle and air sacs. Articular surfaces, particularly of the hock and shoulder joints, become variably thinned to pitted over time (Fig. 21.8). Generally no gross lesions are seen in the upper respiratory tract. In the respiratory form of the disease, airsacculitis may be present.

Turkeys. Swellings of the joints may not be as prominent as in chickens, but fibrinopurulent exudate is frequently present when the joints are opened. Lesions in the respiratory tract are variable.

Microscopic

The histopathology of infectious synovitis (70, 73, 139) in chickens and respiratory disease caused by *M. synoviae* in chickens (43) and turkeys (48, 132) has been described.

The joints, particularly of the foot and hock, have an infiltrate of heterophils and fibrin into joint spaces and along tendon sheaths. The synovial membranes are hyperplastic with villous formation and a diffuse to nodular subsynovial infiltrate of lymphocytes and macrophages (Fig. 21.9). Cartilage surfaces, over time, become discolored, thinned, or pitted. Air sacs may have a mild lesion consisting of edema, capillary proliferation, and the accumulation of heterophils and necrotic debris on the surface, to more severe lesions with hyperplasia of epithelial cells, a diffuse infiltrate of mononuclear cells and caseous necrosis. Other lesions reported to be associated with infectious synovitis are hy-



21.7. Incised swollen foot pad of 8-week-old turkey with granulation tissue and purulent exudate surrounding digital flexors. Similar lesions can be seen in chickens.



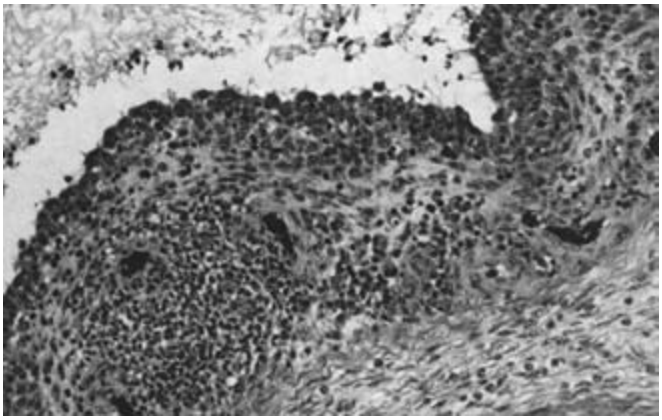
21.8. Ulceration of articular surface of distal tibiotarsus from a chicken with infectious synovitis.

perplasia of the macrophage-monocyte system associated with the sheathed arteries of the spleen; lymphoid infiltrates in the heart, liver, and gizzard; and thymic and bursal atrophy. Cardiac pathology has been described in detail (72).

Pathogenesis of the Infectious Process

There is considerable variation among isolates in their ability to produce disease; many isolates cause little or no clinical disease. Although most recent isolates from the U.S. appear to be of relatively low virulence, clinical infectious synovitis has recently been reported in broilers in California (138). There have been numerous field reports of MS strains of high virulence in Mexico, Argentina, The Netherlands, and Eastern Europe. The pathogenicity and virulence of field isolates have been compared experimentally (56, 89, 103). Significant variability in virulence among strains was found, but no evidence was found for selective tropism for epithelial membranes of the lower respiratory tract to those of the joints, tendon sheaths,and bursae (56). These strains also showed differences in pathogenicity for chick embryos (90), but this did not correlate with virulence for chickens. Potential virulence factors such as hemagglutination and hemadsorption, attachment to cells, and ciliostasis were also studied. Previously found differences in virulence for chickens could not be explained by these factors (91). They concluded that pathogenicity involves attachment and colonization of the upper respiratory tract plus additional unidentified factors associated with systemic invasion and lesion production. Passage in embryos, tissue culture, or broth reduces its ability to produce typical infection. Embryo passage appears to have less effect on pathogenicity than broth passage.

M. synoviae isolated from air sac lesions are more apt to cause airsacculitis, while those isolated from synovia are more apt to produce synovitis (78). Airsacculitis is exacerbated by ND-IB vaccination (77, 140) or any respiratory infection. The severity of the airsacculitis depends on the virulence of the infectious bronchitis virus used in conjunction with MS (58). Air sac lesions are greatly enhanced by cold environmental temperatures (157). Infectious bursal disease causes immunosuppression in chickens, and dual infection with MS results in more severe air sac lesions (49). However, no synergistic effects were seen with dual infections with avian pneumovirus (74) or with *Ornithobacterium rhinotracheale* (164). Nervous signs with lesions of meningeal vasculitis have been seen in MS-infected turkeys displaying severe synovitis (29).



21.9. Hyperplastic synovial membrane with multiple subsynovial lymphoid aggregates from a 7-week-old turkey with infectious synovitis.

Immunity

Chickens exposed intranasally to *M. synoviae* were resistant to subsequent foot pad challenge (117). Chickens immunized intranasally with a temperature-sensitive mutant of MS were protected against airsacculitis for at least 21 wk (107). Prior expo-

sure to MS-H, a temperature-sensitive mutant strain, protected against subsequent challenge with a virulent synovitis-producing field strain (137). Parenteral inoculation of MS frequently overwhelms the bird before adequate resistance can develop. Resistance to lesions induced by MS is bursa dependent (81, 148), while thymus-dependent lymphocytes may be needed for the development of macroscopic synovial lesions (81).

Diagnosis

Isolation and Identification

Positive diagnosis may be made by isolation and identification of *M. synoviae.* Isolation from lesions in acutely infected birds is not difficult, but in the chronic stages of infection viable organisms may be no longer present in lesions. Isolation from the upper respiratory tract is more reliable in chronically infected birds. (For medium and isolation methods, see Growth Requirements.) The fluorescent antibody technique using colony imprints (31) or intact colonies (142) may be used for the identification.

Direct detection of MS DNA in tissues or culture medium utilizing DNA probes has been described (7, 40, 60, 71, 162). It is a simple, rapid method of detection, but sensitivity may not be adequate. Polymerase chain reaction is a simple, rapid, and highly sensitive method of detection of MS DNA in tissues or culture medium (46, 86, 136, 163), and PCR kits (145) are commercially available. The PCR procedures are comparable in sensitivity to isolation and identification (135).

Serology

Antigen is available commercially for the serum plate agglutination (SPA) test. Adequate directions for use are given with each package. Generally 0.02 ml serum is mixed with an equal amount of antigen on a glass plate, which is gently rotated and observed for agglutination. Antigen should be tested with known positive and negative serums each day. Approximately 2–4 wk are required for antibodies to develop in infected birds (116). The SPA test may be insensitive in some instances; Ewing *et al.* (35) reported that the SPA test missed infected commercial layer and breeder flocks that were detected by ELISA.

Nonspecific reactors occur in some flocks when using the SPA test (50, 158), especially in flocks that have been vaccinated with oil emulsion vaccines against various agents. *M. gallisepticum* antigen may be agglutinated on occasion, but reaction is somewhat delayed and usually lower in titer (119). To confirm specificity of the reaction, the HI test is used (146). In a comparison of diagnostic tests for MS, all serological procedures studied exhibited some false-positive activity (39). They concluded that it is not advisable to rely completely on any single serological procedure. In another comparison of diagnostic procedures, PCR was found to be more sensitive than either serology or culture (41).

An indirect immunoperoxidase test utilizing intact *Mycoplasma* colonies as substrate has been utilized to detect antibodies in serum, respiratory secretions, synovial fluids, bile, Harderian gland, oviduct, and yolk (11, 12, 13).

Enzyme-linked immunosorbent assay (ELISA) (55, 123, 126) is commonly used as a diagnostic test and for routine testing of flocks, and may replace serum plate agglutination as the primary serologic test. ELISA kits are available commercially. A semipurified preparation containing antigens in the p46–52 region showed promise as an ELISA antigen (51). A recombinant antigen containing a highly antigenic domain of MSPB (108) has potential as a serodiagnostic reagent (110). The recombinant MSPB antigen from vaccine strain MS-H was more efficient in detecting antibodies in vaccinated chickens than was a similar antigen prepared from strain WVU 1853, suggesting some antigenic variability in that region (112). ELISA has also been used to detect antibodies in the yolk of eggs from commercial layers (52).

Specific antibodies of the IgG, IgM, and IgA classes have been detected in the oviduct and albumen of chickens naturally infected by MS, as well is in their developing embryos (17).

Further confirmation of serologic results may be made by isolation and identification of *M. synoviae* from the upper respiratory tract (134) or by PCR.

Turkeys produce a low level of antibody following respiratory infection; therefore, agglutination may not be effective in determining the *M. synoviae* status of a flock. Significant antibodies develop following foot pad inoculation only (48, 129). Various commercial agglutination antigens vary in their ability to detect agglutinins in turkeys. Under some situations there may be a delayed antibody response in chickens that were proved to be infected by PCR (36). Individual infected turkeys may not develop detectable antibodies(124). Turkeys that were infected systemically developed a strong antibody response; whereas, those birds infected via the upper respiratory tract did not develop circulating antibody, or antibody production was delayed (80). Culture, PCR, and HI testing may be required in some cases to detect infection.

Differential Diagnosis

A presumptive diagnosis may be made on the basis of pale comb, droopiness, emaciation, leg weakness, breast blisters, enlarged foot pads or hock joints, splenomegaly, and enlarged liver or kidneys. Bacteria as causes of synovitis or arthritis must be eliminated by bacteriologic procedures. *Staphylococcus aureus, Escherichia coli,* pasteurellae, and salmonellae may also be present as primary causes of synovitis. *M. gallisepticum* may also be a cause of breast blisters and joint lesions (115, 118).

Fibrosis of metatarsal extensor or digital flexor tendons and lymphocytic infiltration of the myocardium associated with the viral arthritis agent help to differentiate it from *M. synoviae* (93). Serum from viral tenosynovitis-infected chickens does not agglutinate MS antigen, but one must bear in mind that MS agglutinins may be present without obvious joint involvement.

In cases with respiratory involvement, *M. gallisepticum* and other causes of respiratory disease should be eliminated.

Intervention Strategies

Management Procedures

M. synoviae is egg transmitted, and the only effective method of control is to select chickens or turkeys from MS-free flocks.

Most primary breeding stocks are free of infection, and MS-free sources of replacement breeding stocks should be available. Effective biosecurity measures should be used to prevent introduction of the infection.

Outbreaks of MS infection in broilers can often be traced to a specific breeder flock. By the time the infected breeder flock is found, egg transmission may be low or no longer of clinical significance. The decision to slaughter infected parent breeder flocks is often made on an economic basis. If such flocks are kept for egg production, progeny should be hatched separately and isolated from MS-free flocks. Antibiotic treatment of breeders is not effective in eliminating MS, although the level of egg transmission may be reduced.

M. synoviae is susceptible *in vitro* to several antibiotics, including chlortetracycline, danofloxacin, enrofloxacin, lincomycin, oxytetracycline, spectinomycin, spiromycin, tetracycline, tiamulin, tilmicosin, aivlosin, and tylosin (21, 26, 54, 65, 68, 76, 154). In contrast to *M. gallisepticum,* MS isolates appear to be resistant to erythromycin (21, 154). High level resistance to erythromycin and tylosin developed rapidly after low level exposure *in vitro*, but enrofloxacin resistance developed more gradually. No resistance to tiamulin or oxytetracycline was shown (47). Earlier isolates seem to respond more poorly to chlortetracycline than later isolates (121). Generally, suitable medication is of value in preventing airsacculitis or synovitis, but treatment of existing lesions is less effective. Antibiotic medication is not thought to eliminate MS infection from the flock, but in a field study a flock naturally infected with MS was medicated with three treatments of enrofloxacin and later continuous medication with 600 ppm oxytetracycline in the feed. This flock subsequently became MS negative by PCR, suggesting possible clearance of MS from the flock as a result of medication (42). In another study (141), treatment with enrofloxacin at 10 mg/kg in the drinking water for 14 days did not eliminate detection of MS by PCR. In another study, two treatments with enrofloxacin at recommended dose levels did not affect recovery of MS from tracheal swabs, and isolates from the medicated birds exhibited an increased resistance to enrofloxacin as well as substitutions in the topoisomerase IV gene (87).

A summary of data obtained from field and experimental studies indicates that chlortetracycline (50–100 g/ton of feed) given continuously will provide satisfactory control of infectious synovitis in chickens. Higher concentrations (approximately 200 g/ton) are required to control synovitis after infection has occurred. In turkeys, prophylactic levels of 200 g/ton are required. Effectiveness of chlortetracycline may be related to the MS isolate involved (121).

Soluble lincomycin-spectinomycin (2 g/gal of drinking water) is of value in preventing airsacculitis in broilers (53). Tiamulin in the drinking water (0.006–0.025%) has been shown to be effective in preventing airsacculitis and synovitis in chickens (6). Other products have been used, but their value in treatment of MS has not been adequately studied.

Treatment of eggs with antibiotics such as tylosin by egg dipping, or egg inoculation with tylosin and gentamycin (106), or heat treatment (156) of hatching eggs has been used in breeding flocks to prevent egg transmission of MS. Exposure of breeders before the onset of egg production with virulent MS will reduce egg transmission. This should only be used in flocks in which infection will almost certainly occur.

Vaccination

An inactivated, oil emulsion bacterin is commercially available, but its role in the control of MS has not been adequately studied. A live temperature-sensitive MS vaccine strain, MS-H, was selected by mutagenesis of a field isolate from Australia (104). Its safety and efficacy have been established under laboratory (94, 95) and field (96) conditions. Vaccine doses of 4.8×105 ccu/ml were protective (63); immunity was detected after 3–4 weeks postvaccination (62). Factors other than the temperature-sensitive phenotype appear to be involved in the attenuation of the MS-H vaccine strain (113). This vaccine has received wide use in Australia, but it is not licensed in many countries, including the United States.

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Mycoplasma iowae Infection

Janet M. Bradbury and Stanley H. Kleven

Introduction

Mycoplasma iowae has been associated with reduced hatchability and embryo mortality in turkeys. It has also been shown experimentally to induce mortality in turkey and chicken embryos and mild to moderate airsacculitis and leg abnormalities in chickens and turkeys. There have been occasional reports of leg problems in young turkeys associated with natural infection of *M. iowae.*

Economic Significance

Poult hatchability can be reduced by 2–5%.

History

The Iowa 695 strain of avian mycoplasma was isolated in 1955 and characterized by Yoder and Hofstad (75) and was subsequently designated avian serotype I (76). Avian mycoplasma serotypes I, J, K, N, Q, and R were classified into separate groups by Dierks *et al.* (25) and were subsequently characterized as a single, related group (4, 5, 30). The organism was later named *Mycoplasma iowae* (40) and 695 was designated as type strain. Type 8 strains isolated from turkeys in the United Kingdom (70) and North America (10) were also subsequently identified as *M. iowae* (1).

Etiology

Classification

M. iowae is a typical member of the family Mycoplasmataceae, class *Mollicutes*, having characteristic colonial morphology, no cell wall, and a growth requirement for sterol. Its lack of urease distinguishes it from the genus *Ureaplasma* and places it within the genus *Mycoplasma*. Identification at species level is based on serologic reactions. Immunofluorescence of mycoplasma colonies (68) is a rapid and reliable method for identification. Phylogenetic analysis of the 16S rRNA of *M. iowae* places it in the Pneumoniae group along with *M. gallisepticum* and the human pathogen *M. pneumoniae* (73).

Morphology and Staining

As with other mycoplasmas, Giemsa staining or dark-field examination reveals *M. iowae* organisms as coccobacillary but showing some pleomorphism. Ultrathin sections demonstrate that the cells are bound by a plasma membrane and lack a cell wall (40). Some studies suggest the possible presence of an attachment organelle (1) which would be consistent with its phylogenetic placement, since several phylogenetically related organisms possess this feature.

Growth Requirements

Like other mycoplasmas, *M. iowae* has complex growth demands including a requirement for cholesterol. Incubation is usually at 37°C, although some strains grow best at 41° to 43°C (35) and growth occurs either aerobically or with added CO_2 (40). Recovery of *M. iowae* from tissues appears to be more successful by direct plating on agar than by inoculation of broth (3). Although several formulations of mycoplasma medium have been used successfully, the formulation described by Bradbury (13) works well. The presence and quality of yeast extract are im-

portant, and some field isolates of *M. iowae* may be intolerant of certain media components. It is advisable to perform quality control checks on batches of yeast extract and serum using low passage field isolates of the organism.

Colony Morphology

Colonies on agar are characteristic of mycoplasmas with typical fried egg morphology and a diameter of 0.1–0.3 mm (75).

Biochemical Properties

M. iowae ferments glucose, hydrolyses arginine, does not possess urease or phosphatase, and does not produce films and spots or reduce tetrazolium chloride (40). Glucose metabolism is accompanied by oxygen uptake and thereby distinguishes *M. iowae* from some other avian species (69). Unusually for a mycoplasma, it can grow in the presence of 0.5–1.0% bile salts (62). Some strains are reported to hemagglutinate avian red blood cells (25, 75, 76), but the property is not stable. Although *M. iowae* hemagglutinins are variably expressed, the relevant proteins and genes have not yet been characterized (9).

Susceptibility to Chemical and Physical Agents

Resistance of *M. iowae* to disinfectants has not been determined, but it is probably similar to that of other mycoplasmas. *M. iowae* appears to be hardier than *M. gallisepticum* or *M. synoviae* (24). Under experimental conditions strains survived for 5 days or more on feathers and at least 6 days on human hair and several other materials. This may have implications for terminal site disinfection, especially with the existence of an oral/fecal mode of transmission. Practically, however, the organisms appear to be inactivated by proper cleaning and disinfection.

Antigenic Structure and Toxins

The antigenic structure of *M. iowae* is not well characterized, and there appears to be significant antigenic variation among strains (15, 35, 56, 77). Although few differences were seen between SDS-PAGE profiles (56, 77), immunoblotting using monoclonal antibodies revealed considerable diversity between different strains (56). Colony immunoblots probed with a monoclonal antibody demonstrated phenotypic variation of surface antigen in *M. iowae* (28, 60). A surface-exposed protein of 41 kD was identified in *M. iowae*, *M. gallisepticum,* and *M. imitans* giving the first evidence of an antigenic relationship between *M. iowae* and these other two species (59).

Toxins have not been reported for *M. iowae*.

Strain Classification

Antigenicity

In the past, *M. iowae* strains have been loosely classified into one of several serovars (25). *M. iowae* strains give a poor antigenic response in chickens and turkeys in terms of antibodies and little is known about the cellular immune response. When antibodies were raised in chickens to 12 different avian mycoplasma species, it proved to be more difficult with *M. iowae* than any of the others (14). Furthermore, no single antiserum, even from hyperimmunized rabbits, could detect all members of the group by immunofluorescence (23).

Immunogenicity or Protective Characteristics

Little is known about the immunogenicity of *M. iowae*, and it may even be slightly immunosuppressive because experimental infection of day old turkeys resulted in a decreased bursa to body weight ratio and a slightly delayed antibody response to sheep erythrocytes (16). A monoclonal antibody that reacts with a 65 kD polypeptide in *M. iowae* may play a role in cytadhesion (27).

Genetic or Molecular Characteristics

The genome size of *M. iowae* is reported as 1280–1315 kbp, making it one of the largest within the genus. Heterogeneity in the DNA of *M. iowae* strains was reported (23, 77) using restriction enzyme analysis and by restriction fragment length polymorphism using Southern hybridization with 16S rRNA probes (51). Neither of these methods provided clear cut classification of strains.

Pathogenicity

Variability exists in the pathogenicity and virulence of *M. iowae* strains (45, 58, 75). Experimental infection with *M. iowae* causes dose-related mortality in chicken and turkey embryos (20, 34, 54, 58, 75). Under field conditions, it is responsible for embryo mortality and reduced hatchability in turkeys, but some field strains appear to be more embryo lethal than others. The hatchability loss is widely variable and dependent on the extent of vertical transmission. Hatchability losses are not always apparent in eggs from infected turkey breeder flocks. On other occasions, however, the losses can be quite severe and prolonged. The reasons for these differences are unknown but could include the differences in virulence of strains, the incubation conditions, and the susceptibility of breeds of turkeys.

Artificial challenge with *M. iowae* induces mild to moderate airsacculitis in turkeys (25, 58, 75), as well as leg lesions in both chickens and turkeys (17, 19, 22, 75). The severity of lesions appears to vary with the strain. Artificial inoculation of 1-day-old broiler breeder chickens resulted in stunting and poor feathering in addition to leg lesions (18). Few clinical reports, however, describe airsacculitis or leg problems in chickens or turkeys, or embryo mortality in chickens under field circumstances. An outbreak associated with *M. iowae* in commercial turkey poults exhibiting leg weakness and dehydration has been described (71).

Virulence Factors

Virulence factors have not been studied for *M. iowae*.

Pathobiology and Epidemiology

Incidence and Distribution

In addition to North America, *M. iowae* has been reported in Western Europe (39), Eastern Europe (8), India (57), Japan (66), and Taiwan (53). It is presumed to be almost worldwide in distribution, although it has not been detected in Australia.

Natural and Experimental Hosts

The natural host appears to be the turkey, but isolation of *M. iowae* from chickens is not uncommon (8, 75), and it has also been reported in geese (53). In addition, *M. iowae* has been isolated from yellow-naped Amazon parrots (12) and from wild and exotic birds in the United Kingdom (2).

Age of Host Commonly Affected

Turkey embryos in the later stages of incubation are most commonly affected, although any age of live turkey may be infected. Little is known about other hosts, although the mycoplasma was isolated from natural infections of adult chickens (20) and adult geese (53).

Transmission, Carriers, and Vectors

Only avian species are known to be infected with *M. iowae*, although there is one report of its isolation from an apple seed in France (35). Egg transmission occurs in turkeys (54, 75), and it is possible that lateral transmission could occur in the hatchery because the organism is present in the meconium. This is because, unlike other avian mycoplasmas, *M. iowae* exhibits a predilection for the digestive tract (55).

Horizontal transmission may occur, but the organism does not spread rapidly in young flocks. Before achieving reproductive maturity, very few birds may be identified as culture-positive within a flock.

Infection may be spread venereally, and under modern methods of insemination, infected semen may play a role in dissemination (46, 61). After laying begins, following artificial insemination and for a few weeks thereafter, a high percentage of birds may become culture-positive. The organism can be recovered from both cloacal and vaginal sites. Although the infected male may play a role in lateral spread, vaginal contamination following hand contact at artificial insemination may be more important (6). The patterns of vertical transmission have been well characterized (34). Within any infected flock, it is possible to identify individuals that do not lay any infected eggs. Other birds lay only one or a few infected eggs, and the remainder lay many infected eggs. It is the latter group that is important in determining the extent of vertical transmission.

Incubation Period

Embryos infected via the dam usually die from about 18 days of incubation.

Clinical Signs

No clinical signs are usually observed in live turkeys, although one report (71) associates *M. iowae* with leg weakness in young poults, as do occasional further reports from the field. Eggs from infected turkey breeders may have reduced hatchability (usually 2–5%). Affected embryos usually die during the last 10 days of incubation, typically from days 18–24, although death may occur later. Experimentally infected embryos showed a significantly reduced embryo to egg weight ratio (55).

Pathology

Gross

Lesions in affected embryos consist primarily of stunting and congestion, with various degrees of hepatitis, edema, and splenomegaly (54, 55, 75). Sometimes affected embryos exhibit a down abnormality, "swollen down plumule," particularly in severe cases. The chorioallantoic membranes of inoculated turkey embryos are edematous and sometimes hemorrhagic (55). Airsacculitis in inoculated chickens and turkeys is ordinarily mild to moderate and similar to lesions caused by other mycoplasmas (25, 58, 75). Inoculation of day-old poults results in stunting, poor feathering, tenosynovitis, and leg abnormalities including chondrodystrophy, rotated tibia, toe deviations, and sometimes erosion of the articular cartilage of the hock joint and rupture of the digital flexor tendon (22, 75). Similar leg lesions may be observed in experimentally infected chicks, including rupture of the digital flexor tendon (18, 21). Inoculation of turkey poults with *M. iowae* may result in bursal atrophy (16). Lesions are not usually reported under field conditions, perhaps because many infected embryos do not hatch.

Microscopic

The chorioallantoic membranes of inoculated turkey embryos show edema and infiltration with heterophils and mononuclear cells, and the parenchymatous organs show a granulocytopoietic response (55).

After inoculation of day-old poults, lesions of the spleen consist of reticular cells with macrophages, plasma cells, and heterophils in the parenchyma. The bursa of Fabricius has localized congestion with infiltration of plasma cells, heterophils, and reticular cells. Macrophages, lymphocytes, heterophils, and plasma cells are seen in the lamina propria of the duodenum, ileum, and cecal tonsils. Little obvious change is observed in cartilage and tendon except for edema in the tendon sheaths (22). After air sac inoculation of turkey poults, lesions consist of thickened air sacs that contain large numbers of inflammatory cells, primarily lymphocytes. In some areas, lymphoid follicles are observed. Exudate on the mucosal surface contains fibrin and inflammatory cells (58). In experimentally infected broiler breeders, affected joints show acute tenosynovitis, hemorrhage, and tendon fiber degeneration, which is followed later by chronic lymphocyte/plasma cell reaction and tendinous and peritendinous fibrosis (18).

Ultrastructural

Adherence of *M. iowae* to embryo intestinal mucosa was demonstrated in artificial infection (55). Most organisms adhered to the microvilli, which often appeared swollen.

M. iowae organisms have been demonstrated in the crypts of the cloaca and in the secondary mucosal folds of the vagina of the turkey hen by electron microscopy (65).

Pathogenesis of the Infectious Process

Very little is known about the pathogenesis of *M. iowae*. The first step in the invasive process may be attachment to the embryo in-

testinal epithelial surface (55, 65). A 65-kD polypeptide in *M. iowae* may be involved in attachment (27).

In strains that proliferate in the embryo, death probably results from an acute nonspecific inflammation of the chorioallantoic membrane and a granulocytopoetic reaction in the parenchymatous organs (55). Western blots of *M. iowae* reacted positively with antibodies to a 48-kD mycoplasma protein, which has been shown to have immunomodulatory and hematopoietic differentiation activities (36).

Phenotypic variation may play a role in the infectious process by allowing the mycoplasmas to persist despite an immune response. As mentioned previously, there is also a possibility that *M. iowae* may be mildly immunosuppressive (16).

Immunity

Active

A paucity of information on active immunity to *M. iowae* exists, although antibody responses have been observed (75). Equally, very little information on the age susceptibility of turkeys is available. It is difficult or impossible to infect some individuals within a flock of adult breeder turkeys (7). Breeders that become infected and vertically transmit the organism to their eggs usually resolve the infection. This may occur in a few weeks or sometimes can take 2–3 months. Embryo mortality usually subsides immediately before resolution of the infection. That an immune response is involved is suggested by the finding of growth-inhibiting and metabolism-inhibiting antibodies in the serum of these hens (7).

Passive

There appears to be no information on the effects of passive antibodies, including maternally derived antibodies, on *M. iowae.*

Diagnosis

Isolation and Identification of Causative Agent

M. iowae may be present in high numbers in dead embryos (20, 54). After inoculation of turkey poults, it can be isolated from a variety of tissues, especially from the gastrointestinal tract and cloacal swabs, but isolations become less frequent with age, and organisms could not be recovered after 12 weeks (22, 63). Isolation of *M. iowae* from oviduct, semen, and phallus of adult chickens and turkeys has been reported (57, 61, 75). A combined oviduct/cloacal swab was found to be a useful method of detection during the final stages of an eradication program (74). Cotton swabs from the appropriate tissue are streaked on agar plates and incubated for 4–5 days or longer at 37°C. Typical *Mycoplasma* colonies can be identified by immunofluorescence (68), although the use of polyclonal antisera to several different serovars may be needed to cover for antigenic variation (23). Fluorescein-conjugated rabbit antiserum prepared against a cocktail of all the six serovars (I, J, K, N, Q, and R) was used successfully to detect field and laboratory strains (50). Although the reactions of some monoclonal antibodies might be too specific to detect all isolates, antibodies to a 45-kD antigen reacted with all of 22 field isolates tested and showed no evidence of phenotypic variation in immunoblots (67).

Polymerase chain reaction has been developed for direct detection of *M. iowae* DNA (11, 31, 32, 45, 48, 49, 72, 78). One procedure at least (49) has been used successfully to amplify *M. iowae* from swab samples in the field.

A further refinement of the use of PCR, amplified fragment length polymorphism, has been used to differentiate between avian *Mycoplasma* species and has confirmed that high genetic homogeneity exists amongst *M. iowae* strains examined by this method (38).

Arbitrarily primed PCR may be a useful molecular tool for the epidemiological tracing of field strains (26).

Serology

Although agglutination, metabolism inhibition, indirect hemagglutination, and ELISA tests have all been used for experimental infections (45, 64, 75) the serologic response is weak (21, 22), and nonspecific reactions have been a problem with ELISA (44). Thus, there is no reliable serologic test available for use in the field. Growth-inhibiting and metabolism-inhibiting antibodies have been found in the serum of naturally infected breeding turkey hens (7).

Differential Diagnosis

M. iowae infection should be considered in cases of low hatchability in turkeys, especially when there is evidence of late embryo mortality. However, *M. meleagridis* will also be a consideration in this case. None of the lesions seen in embryos can be considered pathognomonic because there can be similar gross lesions in certain nutritional deficiencies, and the down abnormalities may be very similar to those observed when embryos are overheated in the incubator (29). Although it is not recognized as a significant cause of clinical tenosynovitis, *M. iowae* should be considered as a possibility in cases where there is no apparent explanation for leg problems including tenosynovitis, especially in young turkeys.

Intervention Strategies

Management Procedures

M. iowae was eradicated by certain primary turkey breeders in Europe and the United States by a pre-incubation treatment of hatching eggs with enrofloxacin, backed up by cultural monitoring (1).

There is no reliable serologic testing procedure for *M. iowae* to screen commercial flocks. Screening by culture may also be impractical before birds begin production because of the difficulties involved in isolating the organism and the poor horizontal spread. It is often possible, however, to detect infection in toms and hens before the onset of reproduction.

Clean flocks can be maintained free of *M. iowae* infection by preventing fomite transmission. Special attention should be given when birds reach reproductive age, especially during artificial insemination. It should be noted, however, that *M. iowae* does not always appear to be associated with hatchability losses.

Residual site infection is not known to be a problem where effective terminal cleaning and disinfection procedures are employed. The possibility of contaminated fomites should be borne in mind, however, if adequate cleaning is not achieved between successive flocks.

Vaccination

There is no demand for vaccines against *M. iowae*.

Treatment

Treatment of clinical disease in turkeys associated with *M. iowae* has not been an issue because it is not typically associated with clinical disease. Jordan (41, 42) did show the effectiveness of different antibiotics in reducing levels of infection. Attempts have been made, however, to reduce vertical transmission in commercial flocks to alleviate hatchability losses.

M. iowae appears to be more resistant than the other avian mycoplasma pathogens to some of the commonly used antimicrobials, especially tylosin tartrate (37, 47, 52). The quinolone class of antibiotics, particularly enrofloxacin (Bayer), have sometimes been effective when administered to laying hens in the drinking water, early during production. Eggs from medicated turkeys have been shown to be resistant to *in ovo* challenge with *M. iowae* (42). Egg treatment with enrofloxacin has, however, been more commonly employed. Hatching eggs from affected flocks are vacuum dipped in a solution of the antibiotic. This product is not generally available for food animal use in some countries.

Production of a challenge model that induces persistent infection for the purpose of evaluating antimicrobials has been difficult; a procedure involving inoculation of the lung of day-old poults has been recommended for such purposes (43).

Although little is known about development of resistance to antibiotics *in vivo*, *M. iowae* was shown to develop resistance rapidly during culture in subinhibitory amounts of erythromycin and tylosin (33). Some resistance was also found after culture with enrofloxacin, tiamulin or oxytetracycline and resistant mutants occurred more readily and more rapidly with *M. iowae* and all these antibiotics than it did with either *M. gallisepticum* or *M. synoviae*.

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Other Mycoplasmal Infections

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Mycoplasma imitans

Mycoplasma imitans is of interest because of its close relationship to *M. gallisepticum.* It has been isolated from ducks and geese in France and from a partridge in England. *M. imitans* strains share many phenotypic properties with *M. gallisepticum,* including biochemical reactions, hemadsorption, hemagglutination, and presence of an attachment organelle. The original isolates were initially identified as *M. gallisepticum* on the basis of immunofluorescence and growth-inhibition tests. Further serologic studies indicated only a partial relationship to *M. gallisepticum,* and DNA hybridization studies with the type strains of *M. gallisepticum* showed a DNA homology of 40–46% (5, 10). *M. imitans* contains a gene family closely related to the *pMGA* (now *vlhA*) family of *M. gallisepticum* (29), and shares epitopes with *M. gallisepticum* hemagglutinin *vlhA*, pyruvate dehydrogenase *pdhA*, lactate dehydrogenase, and elongation factor Tu (26).

Polymerase chain reaction (PCR) procedures for *M. gallisepticum* which are based on the 16S rRNA gene (16) do not differentiate between *M. gallisepticum* and *M. imitans.* A commercially available PCR kit for *M. gallisepticum* (IDEXX, Westbrook, Maine), however, does differentiate between the two species, as do PCR reactions based on genes for *mgc2*, *gapA*, and LP (17).

M. imitans causes ciliostasis in chicken and duck tracheal organ cultures and has an adherence structure similar to that seen in *M. gallisepticum* (1). It reproduced respiratory disease similar to but somewhat milder than *M. gallisepticum* in red-legged partridges (13). An isolate of *M. imitans* gained virulence on backpassage in turkeys, and reproduced a respiratory disease which was more severe when it was present in a dual infection with rhinotracheitis virus (14). *M. imitans* did not produce signs or lesions when inoculated into chickens, but in a dual infection with infectious bronchitis virus a synergistic effect was seen (15).

Although *M. imitans* has not yet been reported in the United States, and it has not been found in commercial poultry flocks, there is concern about possible misidentification of isolates as *M. gallisepticum* and possible serologic cross-reactions in testing of field flocks.

Mycoplasma gallinarum

M. gallinarum has not been considered to be one of the pathogenic avian mycoplasma species, but there is one report of consistent isolation from air sacs and tracheas from a series of broiler flocks that were having higher than normal condemnations due to airsacculitis. One of those isolates had the ability to induce airsacculitis when given in conjunction with Newcastle disease-infectious bronchitis vaccine (23). It has also been suggested that *M. gallinarum* infection delays the onset of fatty liver syndrome in commercial layers (6). *M. gallinarum* and *M. gallinaceum* are often isolated as contaminants during attempts to isolate pathogenic avian mycoplasmas.

It was originally classified as avian serotype B (8) and was named *Mycoplasma gallinarum* (11). It grows well on all commonly used avian mycoplasma media, and has characteristics common to all mycoplasmas, including cell and colony morphology, absence of a cell wall, and a requirement for cholesterol. It does not ferment glucose, but reduces tetrazolium, is positive for arginine decarboxylase, and exhibits film and spots (2). There is genetic heterogeneity among various strains (9) as measured by RFLP analysis of genomic DNA.

M. gallinarum is ordinarily isolated primarily from chickens, but it may also been found in turkeys (3, 19). It has been isolated from jungle fowl (33), ducks (12), and pigeons (32). It is considered to be worldwide in distribution. *M. gallinarum* is commonly isolated as a contaminant during attempts to isolate *M. gallisepticum* or *M. synoviae,* especially from adult chickens. Isolation of *M. gallinarum* from chicken embryos (3) and demonstration of the organism in oviducts (7, 45) suggest the possibility of egg

transmission. It is readily identified by immunofluorescence of colonies on agar (43). No serologic test is available.

Mycoplasma pullorum

M. pullorum was classified as avian serotype C (8) and was later named *Mycoplasma pullorum* (21). It has been isolated from chickens, quail, partridge, pheasants, and turkeys(30). *M. pullorum* has been isolated from turkey embryos from flocks in France which were experiencing low hatchability and was shown to be pathogenic for chicken and turkey embryos (30). Like other mycoplasmas, *M. pullorum* isolates demonstrate genetic heterogeneity (27).

Avian Ureaplasmas

Ureaplasmas differ from mycoplasmas primarily in their ability to hydrolyze urea (25). There are several reports of isolation of avian ureaplasmas (18, 24). These organisms subsequently received the name *Ureaplasma gallorale* (25). There are no reports of avian ureaplasma isolation in North America.

Very little is known about the pathogenicity. Artificial challenge of chickens produced no clinical signs or macroscopic lesions (24). Turkeys and chickens challenged with a turkey ureaplasma isolated in Hungary developed fibrinous airsacculitis and serologic responses (35). Ureaplasmas were also isolated in Eastern Europe from turkeys that were experiencing problems with reduced fertility (36).

Mycoplasma Infections of Geese

Three serologically and biochemically distinct *Mycoplasma* species were isolated from geese in Europe (38). One of these has been further characterized and named *Mycoplasma anseris* (4); it has been associated with airsacculitis, peritonitis, and embryo mortality (42). Another was subsequently identified as *Mycoplasma cloacale* (39), and the third was designated strain 1220. Two other isolates, strains 1223 and 1225, also represent two additional species isolated from geese (44).

Clinically, strain 1220 has been associated with reductions in egg production and egg transmission, infertility, inflammation of the cloaca and phallus, and lack of weight gain in hatched goslings (37, 39, 40), but proof of etiology is unclear because mixed mycoplasma species were isolated. Strain 1220, on experimental inoculation of goose embryos and day-old goslings, resulted in embryo mortality and reduced growth of young goslings (40). Strain 1220 has also been implicated in a field syndrome of goslings with respiratory and nervous signs (41). More work needs to be done to clarify the role of these mycoplasmas in the field syndromes described.

Mycoplasma Infections of Pigeons

There are three species of *Mycoplasma* primarily associated with pigeons: *M. columbinasale* (21), *M. columborale,* and *M. columbinum* (34). One or more of these *Mycoplasma* species have been isolated from normal birds (3, 20, 31), as well as birds showing signs of respiratory disease (22, 28, 32). An isolate of *M. columborale* reproduced airsacculitis in chickens (28). Medication of pigeons infected with *M. columborale* with tylosin elicited a favorable response (28, 32). Even though there has been isolation of these organisms from birds showing respiratory signs, and there have been favorable responses to medication, there is no conclusive proof that pigeon mycoplasmas are etiologically involved in naturally occurring respiratory disease of pigeons.

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Chapter 22

Clostridial Diseases

Introduction

H. John Barnes

Four clostridial diseases of poultry or game birds are reviewed in this chapter—ulcerative enteritis (UE) caused by *Clostridium colinum*, necrotic enteritis (NE) caused by *C. perfringens*, gangrenous dermatitis (GD) caused by *C. perfringens* or *C. septicum*, and botulism caused by *C. botulinum*. Societal concerns about antimicrobial resistance and perceptions of organic poultry products being of higher quality have resulted in decreased availability and use of growth promoters and coccidiostats. As a consequence, clostridial diseases, especially NE, have greatly increased in occurrence and economic importance (6, 32, 34). A mild or subclinical form of NE that causes significant production losses has been recognized. Definition and criteria for diagnosis of this form of NE are still hazy, but a serological test for determining alpha-toxin antibodies shows promise as both a diagnostic tool and method to monitor NE activity in a flock (17). Some integrated broiler companies also have experienced substantial increases in GD. Collectively NE and GD now rank among the most important causes of disease-related economic loss among many broiler producers.

The complex interactions that result in clinical NE are being unraveled, however, an incomplete understanding of the pathogenesis of NE has hampered development of alternative control methods. Toxins produced by the organisms are responsible for the pathology of most clostridial diseases. Otherwise the organisms are relatively innocuous unless cofactors exist such as dietary ingredients or changes, severe stress, other infectious agents, coccidiosis, or immunosuppressive infections such as infectious bursal disease or chicken infectious anemia. For example co-infections with *C. septicum* and *Staphylococcus aureus* produce greater mortality and more severe GD than infections with either organism alone (33).

Other clostridial species have been isolated from sporadic diseases. *C. chauvoei* was identified in lesions of the comb and livers of chickens in two flocks with complex disease conditions (25) and from intestines and livers of ostriches in a zoological collection with an unusual neuroparalytic disease (18). *C. difficile* caused high mortality in young ostriches (10, 30). Severe enteritis and enterotoxemia due to *C. difficile* occurred in 153 of 160 young ostrich chicks that died in one flock; a second flock experienced a similar disease with high mortality. The organism was isolated in culture and *C. difficile* enterotoxin was confirmed by enzyme-linked immunosorbent assay (10). Hepatitis was a prominent feature in another group of affected ostriches (30). *C. sordelli* also caused sporadic mortality in ostriches (24, 30). *C. novyi* (20) and *C. sporogenes* (21) have been isolated from diseases in chicks. Four colony types of clostridia were recovered, along with *Mycoplasma synoviae*, from inflamed joints of chickens with infectious synovitis (22). Isolated cases of Tyzzer's disease, caused by *C. piliforme* (26), and enterotoxemia associated with *C. tertium* (8) have been confirmed in psittacines and could possibly be found in poultry in the future.

The host range for clostridial diseases continues to expand. NE caused high mortality in egg-laying chickens (7), ostriches (14), and free-living avian species (2, 3, 4). Mortality in psittacines due to UE has been reported for the first time (23).

Clostridium perfringens increasingly is being recognized as a cause of hepatitis and cholangiohepatitis in chickens at processing (13, 16, 27). *C. perfringens*-associated hepatitis (CPH) is the name that has been given to this liver disease. There is an inverse correlation between CPH and flock performance (16). Subclinical NE results in a higher occurrence of CPH at processing (15). CPH also can be found in chicks at hatching (28). Additionally *C. perfringens* has been associated with cellulitis of turkeys (5, 11), gizzard erosions in young replacement layer pullets (9) and broiler chickens (19), and navel infections in neonatal chicks (12). Possible vertical transmission of *C. perfringens* has been shown (29), which is supported by the finding of CPH in newly hatched chicks (28).

Botulism is uncommon but outbreaks characterized by weakness, paralysis, and increased mortality still occur, especially in turkey flocks. The disease tends to repeat on the same farm. Improved molecular diagnostic methods, vaccines, and a model to predict and manage botulism outbreaks in waterfowl may have application for poultry flocks (1, 35, 36). Botulism typically results from ingestion of preformed toxin; however, the toxicoinfectious form of botulism that resulted from caponizing wound contamination by spores of *C. botulinum* has been identified in poultry (31).

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Ulcerative Enteritis (Quail Disease)

Dennis P. Wages

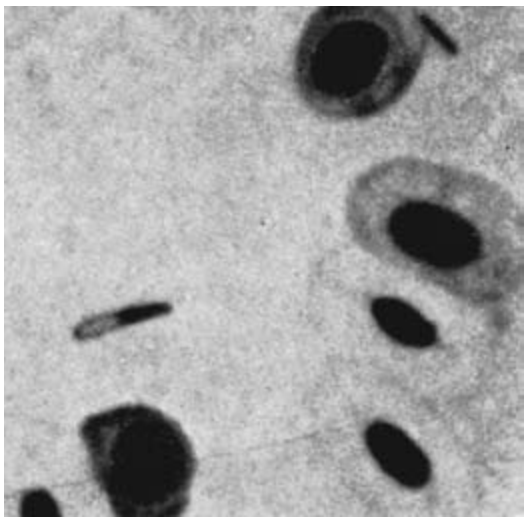
Introduction

Ulcerative enteritis (UE) is an acute bacterial infection in young chickens, turkeys, and upland game birds characterized by sudden onset and rapidly increasing mortality. The disease was first seen in enzootic proportions in quail and was, therefore, named quail disease. It has since been established that many avian species other than quail are susceptible, and the earlier name has been superseded by ulcerative enteritis.

Distribution of UE is worldwide; a number of reports have originated from England (23), Japan (31), Canada (38), Germany (46), and India (26, 48, 49).

Ulcerative enteritis is an important disease problem in some concentrated poultry-raising areas (10) and is a threat to game birds either in confinement or in the wild.

Infection of humans has not been reported.



22.1. Blood smear from quail with UE. Note two bacteria, one of which has a subterminal spore. (M. C. Peckham)

History

Quail disease was first reported in the United States in 1907 (37). Several scattered outbreaks in quail and grouse (1, 20, 32, 33, 34, 42) were reported during the next 2 decades. Subsequently, infection in wild and domestic turkeys (11, 47) was discovered. Other avian species found to be susceptible included pigeons (21), chickens (22, 47), robins (50), pheasants, blue grouse, partridges (44), California quail (12) and lories (43). Genetic differences in susceptibility to ulcerative enteritis have been described in Japanese quail (15). Chronologic events leading to isolation and precise identification of the etiologic bacterium are detailed by Bass (2, 3), Peckham (39, 40), and Berkhoff *et al*. (7).

Etiology

Classification

Ulcerative enteritis is caused by a species of *Clostridium* named *Clostridium colinum* (5, 8). On the basis of 16S rRNA sequence analyses, *C. colinum* has been placed into subcluster XIV-b with 6 other *Clostridium* spp. It is most closely related to *C. piliforme,* the noncultured causative agent of Tyzzer's disease (14).

Initially, a gram-positive, pleomorphic, aerobic, nonmotile bacterium isolated from the liver of a diseased quail was used to reproduce UE in quail. The organism originally was identified as *Corynebacterium perdicum.* It did not grow on solid media and grew poorly in fluid media. On subculture, the organism quickly lost virulence (35). Subsequently, a gram-negative, anaerobic bacillus was isolated from the intestine and liver of infected quail. Feeding quail thioglycolate broth cultures reproduced the clinical syndrome (3).

Peckham (39, 40) reported isolation of a gram-positive, anaerobic, spore-forming rod following yolk sac inoculation of chick embryos. This organism produced UE lesions in inoculated quail. It was re-isolated from inoculated quail, fulfilling Koch's postulates. Similar anaerobes were isolated from chickens and turkeys affected with UE, and it was established that UE in chickens, turkeys, and quail was caused by the same organism (40). Berkhoff *et al*. (8) cultured the etiologic anaerobe on solid media, which allowed the study of its biochemical characteristics.

Morphology and Staining

C. colinum is a gram-positive 1 × 3–4 μm bacillus that occurs singly as a straight or slightly curved rod with rounded ends. Sporulation is rarely seen in artificial media, but if spores are present, they are oval and subterminal. Sporogenic cells are much longer and thicker than nonsporing cells (Fig. 22.1).

Growth Requirements

The organism is fastidious in its growth requirements, needing an enriched medium and anaerobic conditions. The best medium for isolating *C. colinum* is tryptose-phosphate agar (Difco) to which 0.2% glucose and 0.5% yeast extract are added. The pH is adjusted to 7.2, and the medium then is sterilized by autoclaving. After cooling to 56°C, 8% horse plasma is added, and the medium is poured into Petri dishes. Prereduced plates are inoculated with material from liver lesions and incubated anaerobically for 1–2 days at 35–42°C (19); colonies are 1–2 mm in

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diameter, white, circular, convex, and semitranslucent and have filamentous margins. Growth in broth media, prepared as stated previously but without agar, can be detected as early as 12–16 hours postinoculation (PI). Actively growing cultures produce gas. Gas production continues for no more than 6–8 hours, after which growth settles to the bottom of the tube (5). Subcultures should be made from actively growing broth cultures still producing gas; later transfers may be unsuccessful.

Biochemical Characteristics

The following carbohydrates are fermented: glucose, mannose, raffinose, sucrose, and trehalose. Fructose and maltose are weakly fermented. Mannitol is fermented only by some strains, one of which is the type strain, ATCC 27770. Carbohydrates not fermented are arabinose, cellobiose, erythritol, glycogen, inositol, lactose, melezitose, melibiose, rhamnose, sorbitol, and xylose. Fermentation products of this organism are acetic and formic acids (5, 8).

Esculin is hydrolyzed. Starch hydrolysis is usually negative; only 2 strains have been found to cause starch hydrolysis. The type strain does not hydrolyze starch. Nitrite and indole are not produced. Milk is unchanged, and casein is not digested. Good growth occurs in chopped meat carbohydrate (CMC) broth. Pyruvate and lactate are not used. Gelatin is not liquefied. Catalase, urease, lipase, and lecithinase are not produced.

C. colinum resembles *C. difficile* most closely. These 2 organisms can be differentiated on cultural characteristics. *C. difficile* hydrolyzes gelatin and is unable to ferment raffinose, whereas *C. colinum* is inactive on gelatin and readily ferments raffinose (19).

Susceptibility to Chemical and Physical Agents

The anaerobe, by virtue of its spore-forming characteristic, is extremely resistant to chemical agents and physical changes. Spores of *C. colinum* are resistant to octanol and chloroform (8). Yolk cultures have remained viable after 16 years at –20°C and survive heating at 70°C for 3 hours, 80°C for 1 hour, and 100°C for 3 minutes (40).

Pathogenesis and Epidemiology

Incidence and Distribution

Ulcerative enteritis is worldwide in its distribution and affects a wide variety of avian species. Even though a common infection recognized in quail, chickens and turkeys can be affected worldwide.

Natural and Experimental Hosts

Ulcerative enteritis is found in a wide range of avian hosts, but quail are undoubtedly among the most susceptible species. Natural infections have been found in the following: bobwhite quail (*Colinus virginianus*), California quail (*Lophortyx california*), Gambel quail (*L. gambelii*), mountain quail (*Oreortyx picta*), scaled quail (*Callipepla squamata*), and sharp-tailed grouse (*Pedioecetes phasianellus*) (37); ruffed grouse (*Bonasa umbellus*) (32, 33); domestic turkeys (*Meleagris gallopavo*) and chickens (*Gallus gallus*) (18, 47); European partridge (*Perdix perdix*) and wild turkeys (*M. gallopavo*) (18); chukar partridge (*Alectoris graeca*) (44); pigeons (*Columba livia*) (21); pheasants (*Phasianus colchicus*) and blue grouse (*Dendragapus obscurus*) (12); and crested quail (*L.c. californicus*) (23). An outbreak of UE in robins (*Turdus migratorius*), confirmed by isolation of *C. colinum* from the liver, was the first evidence that UE could affect passerine birds (50). UE has also been confirmed in lories (*Trichoglossus* spp.) and *Eos* spp. (43).

Although chickens frequently are infected naturally, experimental infections are difficult and can be readily produced only in quail (9). Ulcerative enteritis is more frequently seen in young birds. It occurs in chickens 4–12 weeks (40), turkeys 3–8 weeks (11), and quail 4–12 weeks of age. An outbreak has been reported in adult quail (27).

Outbreaks in chickens often accompany or follow coccidiosis, chicken infectious anemia, infectious bursal disease, or stress conditions. The importance of coccidiosis in outbreaks of UE in chickens was confirmed by producing UE in 5-week-old chickens previously infected with *Eimeria brunetti* and *E. necatrix* but not with either one alone (16).

Age of Host Commonly Affected

Quail of all ages can be affected by UE, however, the young growing quail are more commonly observed with infection.

Transmission

Under natural conditions, UE is transmitted through droppings; birds become infected by ingesting contaminated feed, water, or litter. The organism produces spores, resulting in permanent contamination of premises after an outbreak has occurred. Oral administration of at least 10^7 viable cells of *C. colinum* is required to experimentally reproduce UE in quail (8).

The carrier status of recovered birds or survivors in a flock has not been studied critically. Chronic carriers, however, have been considered to be one of the most important factors in perpetuation of UE and a complement-fixation (CF) test to detect them has been used (36).

Incubation Period

Following experimental infection in quail, the acute form of UE results in death within 1–3 days. The course of the disease in a flock generally lasts about 3 weeks, with peak mortality occurring 5–14 days PI.

Clinical Signs

Birds dying from acute disease may exhibit no premonitory signs. They are usually well muscled and fat and have feed in the crop. Quail often exhibit watery, white droppings. As UE progresses, infected birds become listless and humped up, with eyes partly closed and feathers dull and ruffled. Extreme emaciation with atrophy of pectoral muscles is seen in birds affected 1 week or longer.

Morbidity and Mortality

Mortality in young quail may be as high as 100% in a matter of a few days. Chicken losses typically range from 2–10%.

Pathology

Gross

Acute lesions in quail are characterized by marked hemorrhagic enteritis in the duodenum. Small punctate hemorrhages may be visible through the serosa in the intestinal wall. Ulcerations may be extensive enough to erode through the intestinal wall, perforating the intestines resulting in peritonitis.

In birds that have survived infection for several days, inflammatory changes are followed by necrosis and ulceration, which may occur in any portion of the intestine and ceca. Early lesions are characterized by small yellow foci with hemorrhagic borders, which may be seen on serosal and mucosal surfaces. As ulcers increase in size, the hemorrhagic border tends to disappear. Ulcers may be lenticular or roughly circular in outline, sometimes coalescing to form large necrotic, diphtheritic patches. The lenticular shape is more common in the upper portion of the intestine. Ulcers may be deep in the mucosa; in older lesions, they may be superficial and have raised edges. Ulcers in ceca may have a central depression filled with dark-staining material that cannot be rinsed off. Perforation of ulcers frequently occurs, resulting in peritonitis and intestinal adhesions. Gross lesions in the intestine are shown in Figure 22.2A,B.

Liver lesions vary from light yellow mottling to large, irregular yellow areas along the edges. Other liver lesions are disseminated gray foci or small, yellow circumscribed foci, sometimes surrounded by a pale yellow halo (Fig. 22.2F). The spleen may be congested, enlarged, and hemorrhagic. Gross lesions are absent in other organs. Peckham (40) described an unusual lesion of UE in turkeys characterized by a necrotic, diphtheritic membrane occupying the middle third of the intestine. This combination of necrosis and sloughing of intestinal mucosa appeared similar to lesions produced by *E. brunetti* infection in chickens.

Microscopic

For a description of the histopathology of UE in quail, see reference 16. Intestinal sections from acute cases reveal desquamation of mucosal epithelium, edema of intestinal wall, vascular engorgement, and lymphocytic infiltration. The lumen of the intestine contains desquamated epithelium, blood cells, and fragments of mucosa. Early ulcers consist of small hemorrhagic, necrotic areas involving villi and extending into the submucosa. Cells adjacent to these areas exhibit coagulation necrosis with karyolysis and karyorrhexis. Lymphocytic and granulocytic infiltration occurs adjacent to necrosis. Small clumps of gram-positive bacteria are often present in necrotic tissue. Older ulcers appear as thick masses of granular, acidophilic, coagulated serum proteins mixed with cellular detritus and bacteria. Infiltrations of granulocytes and lymphocytes surround the ulcer. Microscopic pathology of the intestine is illustrated in Figure 22.2C,D,E. Small blood vessels near ulcers and in liver occasionally are occluded by thrombi and bacteria. Liver lesions consist of poorly demarcated foci of coagulative necrosis, with minimal inflammatory reaction and occasional intralesional, gram-positive bacterial colonies, scattered throughout the parenchyma (23) (Fig. 22.2F,G,H).

Pathogenisis of the Infectious Process

Pure cultures of *C. colinum* grown anaerobically were highly pathogenic for quail following oral inoculation. The experimental disease appeared either in an acute form with birds dying around 3 days PI or in a more chronic form with deaths occurring after 1–2 weeks (7).

Immunity

Active Immunity

Active immunity seems to develop in birds that recover from naturally occurring infections. When survivors of a UE outbreak were subsequently challenged, no noticeable effect was seen (28), whereas 85% of similarly challenged susceptible controls died. It has been observed, however, that survivors in groups treated with antibiotics may remain highly susceptible to infection (30, 41).

Diagnosis

Diagnosis of UE can be made on the basis of gross postmortem lesions. The presence of typical intestinal ulcerations accompanied by necrosis of the liver and an enlarged, hemorrhagic spleen suffices for clinical diagnosis. As an aid in diagnosis, necrotic liver tissue can be crushed between two slides, fixed by heat, and stained by Gram's method. Large, gram-positive rods, subterminal spores, and free spores can be seen. If necessary, *C. colinum* can be isolated from liver or spleen (see "Isolation and Identification of Causative Agent").

A fluorescent antibody (FA) has been developed and found to be highly specific for diagnosis of UE; correlation between a presumptive diagnosis based on gross lesions and the FA test was 100% (6).

Isolation and Identification of Causative Agent

A clinical diagnosis of UE can be confirmed by isolation and identification of *C. colinum.* Because the organism is often present in the liver in pure culture, isolation from liver rather than from ulcerative, intestinal lesions is recommended. *C. perfringens* may be present as a secondary invader but is easy to recognize (7, 19) (see "Growth Requirements").

Serology

An agar gel immunodiffusion test has also been used for diagnosis of UE (4). Soluble bacterial antigens that reacted with antisera prepared against *C. colinum* were found in high concentrations in intestinal contents. These antigens were identical to bacterial antigens present in culture filtrates of *C. colinum.* Antigens were not, however, species-specific, as some strains of *C. perfringens* types A and C have cross-reacting antigens. Cross-reactivity among clostridial species makes this test unreliable for diagnostic purposes.

Differential Diagnosis

Among similar diseases that must be differentiated from UE are coccidiosis, necrotic enteritis, and histomoniasis. Frequently, coccidiosis in chickens, turkeys, and pheasants precedes or occurs concurrently with UE (Fig. 22.3). Both diseases may be



22.3. Combined UE and *Eimeria brunetti* coccidial infection in the intestine of a chicken. Note small ulcers in ceca and rectum. Diphtheritic membrane is due to coccidial infection. (M. C. Peckham)

present in the same or different specimens submitted for diagnosis (11, 12, 16, 39). It is imperative that a differential diagnosis between coccidiosis and UE be made because medication for each disease is distinct. Furthermore, both diseases may occur simultaneously, necessitating use of 2 different medications.

A condition initially described as necrotic enteritis frequently occurs in broilers in densely populated areas. Although there was much controversy that UE and necrotic enteritis are the same disease, it has been conclusively demonstrated (17) that they are distinct. Gross and histopathologic differentiation of necrotic enteritis and UE has been described (24) (see "Necrotic Enteritis").

Histomoniasis produces caseous cores in ceca and necrotic areas of varying size in the liver. This combination of cecal and liver lesions seen in chickens, turkeys, and other gallinaceous birds makes it imperative that cecal ulcerations and liver necrosis of UE be distinguished from histomoniasis. An enlarged hemorrhagic spleen and intestinal ulcerations are characteristic of UE. Histologic examination of the liver or ceca will reveal histomonads (see Chapter 28).

Intervention Strategies

Management Procedures

Because the infectious organism is in the droppings and remains viable indefinitely in litter, it is recommended on problem farms to remove contaminated litter and use clean litter for each brood. In chickens, avoid stresses caused by overcrowding, keep coccidiosis under control, and use preventive measures against viral diseases, which may act as stressors and/or cause immunosuppression.

Game farm managers should exercise caution with regard to overgrazing ranges or overcrowding birds. Placing birds on 0.5-inch wire mesh is recommended on farms where the disease is a problem. Survivors of an outbreak may be carriers and should not be mixed with unexposed birds.

Treatment

Early attempts to use sulfonamides for treatment of UE were unsuccessful (13, 44). Streptomycin administered by injection or in feed or water has prophylactic and therapeutic value against UE in quail. Streptomycin at a level of 60 g/ton of feed or 1 g/gal of water gives complete protection when administered prophylactically (27, 28, 29, 30, 41). Addition of 100 g bacitracin/ton feed provides protection (41). Bacitracin methylene disalicylate can be used at 200 grams per ton for control of ulcerative enteritis in quail. Other chemotherapeutics reported to have efficacy for controlling UE in quail include furazolidone, chlortetracycline (CTC) (41), penicillin, ampicillin (31), and tylosin (25). Kondo *et al.* (31) tested the *in vitro* sensitivity of *Clostridium colinum* to 19 antimicrobial agents.

Ulcerative enteritis can be prevented and/or controlled through medication by either drinking water or feed.

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Necrotic Enteritis

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Introduction

Definition and Synonyms

Clinical necrotic enteritis (NE) can be defined as a disease of primarily young chickens, caused by infection with, and toxin production by, *Clostridium perfringens* type A and type C. The clinical infection is characterized by sudden onset, high mortality and necrosis of the mucous membrane of the small intestine. Since its first description, sub-clinical (121) and mild (69) forms of the disease have been described. The disease is also known as clostridial enteritis, enterotoxemia and rot gut.

Economic Significance

There have been very few studies that have evaluated the economic significance of the clinical manifestation of NE. The costs associated with NE prevention have been estimated to be approximately $0.05 per broiler in the U.S. (130). While it is difficult to determine the prevalence of the mild form of the infection, it has been shown to impair growth rate and feed conversion (81) and cause higher condemnation rates in broilers due to resulting hepatitis (80). Flocks with high levels of *C. perfringens*-associated hepatitis had 25–43% poorer performance than flocks with low levels of *C. perfringens*-associated hepatitis (81). In countries where the practice of using antibiotics in the feed to enhance growth has been discontinued, the incidence and, therefore, the economic significance of subclinical and clinical NE have increased (54).

Public Health Significance

Clostridium perfringens type A and type C, in addition to producing toxins which can induce NE in poultry, also produce enterotoxins at the moment of sporulation which can produce foodborne illness in humans. Two distinct diseases are induced by these subtypes; type A *C. perfringens* produces diarrhea and type C *C. perfringens* produces necrotic enteritis in humans (131). High percentages of *C. perfringens*-positive carcasses have been reported following processing (28, 92) and outbreaks of type A food poisoning traced to consumption of chicken have been reported (62, 109). Although type C food poisoning is a much more severe disease in humans and type C *C. perfringens* can be found in poultry, it is not considered to be a major foodborne concern because of the very low prevalence of the disease in humans (131). Of concern is the potential for incidence of C. *perfringens*-induced foodborne illnesses to increase with the worldwide trend of removing growth enhancing antibiotics from poultry rations. The removal of these compounds, most of which have anti-clostridial activity, has led to an increase in clinical and sub-clinical NE and hepatitis in broilers (54) and will, no doubt, also increase the carriage of *C. perfringens* on broiler carcasses throughout processing.

History

Necrotic enteritis in domestic chickens was first described by Parish in 1961 (101, 102, 103) who reproduced the disease with a strain of *Clostridium welchii* (*C. perfringens*). It subsequently has been reported from most areas of the world where poultry is produced (7, 13, 22, 23, 66, 73, 77, 94, 95, 129). *C. perfringens* has also been associated with NE in turkeys (38, 39, 50).

Etiology

Strain Classification and Toxin Production

The etiologic agent of NE is a Gram-positive, spore-forming anaerobe; *C. perfringens* type A (3, 9, 14, 72, 78, 98, 110, 128, 134) and type C (32, 72, 95, 103, 110, 112).

Both of these strains are capable of producing various toxins and enzymes which are responsible for the associated lesions and clinical signs. Specifically, alpha-toxin produced by *C. perfringens* types A and C and beta-toxin produced by *C. perfringens* type C are believed responsible for the production of the intestinal mucosal necrosis commonly associated with NE (5, 46, 98, 120).

Healthy and diseased birds may harbor *Clostridium perfringens* type A within their intestines although it is not clear whether isolates from birds with NE produce significantly larger quantities of alpha-toxin than isolates from birds that do not have NE (52, 61). Using an immunoglobulin G anti-alpha-toxin ELISA test, a significant relationship between antibody titer to alpha-toxin and sub-clinical NE as well as an association between antibody titer and the occurrence of *C. perfringens*-associated hepatitis at slaughter has been demonstrated (83).

The quantity of alpha-toxin produced by different isolates appears to be determined by the regulation of a specific gene, *cpa* (115). Expression of *cpa* is thought to be influenced by inducers within the intestinal micro-environment**.** The stimulus for production of these inducers, however, is unclear (107) but it has been speculated that it may be based on quorum sensing—the method through which bacteria produce, secrete, detect and respond to signaling molecules which accumulate in the extracellular environment and influence gene expression (89)**.** Whether expression of *cpa* by *C. perfringens* is down-regulated in the healthy gut, or in response to inducers, is up-regulated to initiate enteric disease is unclear at this time (89).

Morphology and Staining

C. perfringens can be isolated readily on blood agar plates incubated anaerobically at 37°C overnight. *C. perfringens* colonies on blood agar (with rabbit, human, or sheep blood) are surrounded by an inner zone of complete hemolysis and an outer zone of discoloration and incomplete hemolysis and are composed of short to intermediate, gram-positive rods without spores.

Growth Requirements and Biochemical Properties

Positive identification of the organism is made by inoculation of differential media (1). Most strains ferment glucose, maltose, lactose, and sucrose; do not ferment mannitol; and variably ferment salicin. Principal products of fermentation are acetic and butyric acids. Gelatin is hydrolyzed; milk is digested; and no indole production occurs. Growth on egg yolk agar demonstrates the presence of lecithinase and the absence of lipase production. Subculturing on egg yolk agar plates, one-half of which have been spread with *C. perfringens* antitoxin, and incubating anaerobically overnight will produce a zone of precipitation around colonies on control sides of the plate and little or no precipitation on sides spread with antitoxin (1).

Pathobiology and Epidemiology

Natural and Experimental Hosts

Naturally occurring outbreaks of NE have been reported in chickens from 2 weeks to 6 months of age. A majority of reports of NE have been in 2 to 5-week-old broiler chickens raised on litter (7, 13, 48, 57, 66, 77, 93, 95, 129). Cases of necrotic enteritis have also been reported in 3 to 6-month-old commercial layers raised in floor pens (22, 73), 12 to 16-week-old caged-reared commercial layer replacements (20, 45), and mature commercial layers in cages (36). Necrotic enteritis has also been reported in turkey poults (40), 7 to 12-week-old turkeys (50), and turkeys with concurrent ascarid infection (99) or coccidiosis (38).

Necrotic enteritis has been reproduced experimentally in chickens (9, 25, 51, 55, 56, 104, 105), turkeys (43), and Japanese quail (32). In broiler chickens, the incidence of the disease can be from 1.3–37.3% and as high as 62.0% in specific-pathogen-free chicks (9). Necrotic enteritis can be reproduced by rearing chickens on litter in facilities where the disease has previously occurred (55, 56, 86, 133); feeding feed contaminated with *C. perfringens* (78, 128); administering vegetative cultures of *C. perfringens* intravenously (16), orally (16), or into the crop (9); administering intraduodenally broth cultures of *C. perfringens* (3), bacteria-free crude toxins of *C. perfringens* (4), or a combination of *C. perfringens* and its toxins (5, 10); or by dosing chickens with sporulated oocysts of *Eimeria* spp. and feeding vegetative cultures of *C. perfringens* or *C. perfringens*-contaminated feed (2, 8, 9, 10). Others have combined several predisposing factors (dietary inclusion of wheat and fishmeal along with a coccidial and clostridial challenge) to reproduce the disease (136). Immunosuppression due to IBDV challenge has been shown to make the disease more severe (90).

Transmission, Carriers, and Vectors

C. perfringens can be found in feces, soil, dust, contaminated feed and litter, or intestinal contents (26, 28, 29, 72, 74). In various outbreaks of NE, contaminated feed (23, 45, 134) and contaminated litter (133) have been incriminated as sources of infection. Domestic flies have been shown to be a mechanical vector, and perhaps a biological vector as well, in a cage-layer facility that experienced a necrotic enteritis outbreak (36).

C. perfringens can be disseminated through commercial hatcheries to broiler farms (26, 27, 28, 29) and has been isolated from eggshells, hatchery fluff and chick box pads (27). Other studies, using ribotyping, have confirmed the vertical dissemination of *C. perfringens* from commercial breeder farms to the hatchery, broiler farms and, ultimately, the processing plant (29).

Clinical Signs

Clinical signs in naturally occurring outbreaks include marked to severe depression, decreased appetite, reluctance to move, diarrhea, and ruffled feathers (7, 15, 57, 77, 95, 101, 129). Clinical illness is very short; often birds are just found acutely dead without any outward signs of disease.

Pathology

Gross

Gross lesions in naturally occurring outbreaks usually are confined to the small intestine, primarily the jejunum and ileum (Fig. 22.4A) (7, 15, 57, 95, 129); however, cecal lesions have been described (79). Intestines are often friable and distended with gas. The mucosa is lined by a loosely to tightly adherent yellow to green pseudomembrane that is often described as having a "Turkish towel" appearance (Fig. 22.4C). Flecks of blood have been reported, but hemorrhage is not a prominent feature. Experimentally, gross lesions characterized by a gray, thickened mucosa in the duodenum and jejunum may be observed as early as 3 hours following inoculation of *C. perfringens* (3). By 5 hours, there is necrosis of the intestinal mucosa, which progresses over time to a severe fibrinonecrotic enteritis with formation of a diphtheritic membrane (9, 112). Hepatitis, characterized by swollen, tan colored livers with necrotic foci (40) in addition to cholecystitis (100) has been reported in association with classical and subclinical NE infections (80, 81).

A more mild form of the disease has been described characterized by focal areas of intestinal mucosal necrosis, hepatic necrosis and impaired performance with or without clinical signs of the infection (68, 80, 81).

Microscopic

Microscopic changes in natural outbreaks are characterized primarily by severe necrosis of the intestinal mucosa with an abundance of fibrin admixed with cellular debris adherent to the necrotic mucosa (Figs. 22.4B,D) (15, 57, 79, 95, 129). Initial lesions develop at the apices of villi and are characterized by sloughing of epithelium and colonization of the exposed lamina propria with bacilli, accompanied by coagulation necrosis. Areas of necrosis are surrounded by heterophils. Progression of lesions usually occurs from villi apices to crypts. Necrosis may extend into the submucosa and muscular layers of the intestine. Numerous large bacilli often are observed attached to cellular debris. In birds that survive, regenerative changes consist of crypt epithelial cell proliferation with a corresponding increase in mitotic figures. Epithelial cells are primarily cuboidal, with a relative decrease in goblet and columnar epithelial cells. Villi are relatively short and flat. In many outbreaks, various sexual and asexual stages of coccidia are also found in the intestine (57, 79, 95).

Microscopic changes after experimental inoculation of *C. perfringens* occur as early as 1 hour following challenge and consist of slight edema and dilation of vessels in the lamina propria, sloughed epithelial cells in the intestinal lumen, and occasional heterophils and mononuclear cells in the lamina propria (3). By 3 hours, marked edema, resulting in the detachment of the epithelial cell layer from the lamina propria, mostly at the apex of villi, has occurred. Mononuclear cell infiltration of the lamina propria is more marked than earlier. At 5 hours, there is marked coagulation necrosis of the epithelial cell layer and lamina propria at villous tips, resulting in villus shortening. Colonization of organisms may be prominent on necrotic tissues and apices of exposed lamina propria. Blood vessels are very congested, occasionally occluded by hyaline thrombi. By 8–12 hours, there is massive necrosis of villi, in some instances reaching to the crypts, characterized by areas of amorphous eosinophilic-staining material and cell nuclei. Fibrin and cellular debris are present in the lumen. Ultramicroscopically, the most prominent changes of the luminal cell membrane are loss of vesiculation and complete loss of microvilli (67). These changes occur most prominently in necrotic parts of the intestinal mucosa in close proximity to individual *C. perfringens* bacterium and further suggests that hydrolysis of epithelial cell membranes by bacterial toxins is important in the pathogenesis of NE.

Histopathologic lesions of the liver include bile duct hyperplasia, fibrinoid necrosis, cholangitis and focal granulomatous inflammation (80, 100, 108).

Pathogenesis of the Infectious Process

The pathology associated with NE is a result of the alpha- and beta-toxins produced and released by *C. perfringens* within the mid-intestine (4). There continues to be debate over the specific events that initiate toxin production and the importance of relative numbers of clostridia within the intestine in healthy and diseased birds. Some studies have found *C. perfringens* to be the principal obligate anaerobic bacterium in the intestinal tract of healthy chickens (65, 113); whereas others have reported it only sporadically and in low numbers from the small intestine of normal chickens ranging in age from recently hatched to 5 months of age (11, 12, 111, 117, 126). It appears that the make-up of the intestinal population of *Clostridium perfringens* is determined by the health status of the bird. In flocks experiencing necrotic enteritis, isolates tend to be clonal within a flock with different diseased flocks having different clonal populations. Healthy flocks, on the other hand, have more diverse populations of *C. perfringens* isolates (42, 52, 96).

Although the events that lead up to the production of toxin remain unclear, what is clear is the ability of the toxins to induce the lesions and clinical signs characteristic of NE. Alpha-toxin is a phospholipase C sphingomyelinase that hydrolyzes phospholipids and promotes mucous membrane disorganization (97, 131) which then stimulates the arachadonic acid cascade to induce the production of inflammatory mediators like leukotrienes, prostacyclin, platelet-agglutinating factor and thromboxane (21, 127). These mediators lead to contraction of blood vessels, aggregation of platelets and myocardial dysfunction, leading to acute death (131). Beta-toxin induces hemorrhagic necrosis of the intestinal mucosa characteristic of the disease (53, 76).

Contributing Factors

Infection with mid-intestinal species of coccidia is a major predisposing factor for NE (2, 6, 8, 9, 10, 57, 112). Colonization of the small intestine by *Eimeria* sp. may lead to intestinal mucosal damage which may then, in turn, provide natural substrates (plasma proteins) required for *C. perfringens* proliferation (131).

Management factors which may influence the development of NE include the use of high fiber litter (128), bird stocking density and programmed feed changes (moving from starter rations to grower rations, for example) which may create intestinal stress (89). It has also been reported that the occurrence of NE may result from a complex relationship between cereal grain ration (wheat/barley/maize), dietary animal protein level, and seasonal effects (70).

Manipulating the diet can affect the population of *C. perfringens* in the intestinal tract (118) and may also increase the rate of fecal shedding of *C. perfringens* (26), suggesting that *C. perfringens* numbers within the intestinal tract and the onset of intestinal clostridial disease in chickens may be precipitated by the nature of the ration (95, 106). Inclusion of cereal grains in the ration like wheat (18), barley (68) or rye (70, 123) that are rich in water-soluble non-starch polysaccharides can predispose or exacerbate outbreaks of NE. In chickens fed wheat diets, the severity of NE lesions was reduced with the addition of dietary fiber and complex carbohydrates (17).

Additionally, rations containing high levels of protein, specifically protein derived from animal sources, may predispose birds to necrotic enteritis (37, 66, 70, 128). More specifically, it appears that the higher relative glycine content in animal-derived proteins relative to plant-derived proteins may trigger the proliferation of *C. perfringens* and the up-regulation of the genes controlling toxin production (34, 37, 135).

The mechanisms through which these predisposing factors enhance the growth of *C. perfringens* and the incidence of NE are unclear. These factors create conditions which stimulate the secretion of intestinal mucus which then induce the proliferation of mucolytic bacteria within the intestinal lumen (24). Mucolytic bacteria provide substrates favorable for the proliferation of *C. perfringens*.

Genetic resistance to NE influenced by major histocompatibility genotype and background genome has also been suggested (116).

In turkeys, NE has been reported in association with coccidiosis (38), ascaridiasis (99), and clinical hemorrhagic enteritis (39). Gender also appears to be a risk factor for NE, as significantly more male turkey flocks than female flocks were diagnosed (39).

Diagnosis

Isolation and Identification of Causative Agent

Diagnosis of NE can be made based on typical gross and microscopic lesions and isolation of the causative agent. In field cases of NE, *C. perfringens* can be isolated readily from intestinal con-

tents, the scrapings of intestinal wall, or hemorrhagic lymphoid nodules by anaerobic incubation overnight at 37°C on blood agar plates (44). Identification of *C. perfringens* can be done as described under "Etiology." Some commercially available media may not be formulated adequately for selective cultivation and enumeration of *C. perfringens* unless used in combination with other tests for specific identification (33).

Using a sandwich ELISA technique for screening, a difference in the number of *C. perfringens* and the amount of toxin present when comparing sick and healthy birds was demonstrated (87). A polymerase chain reaction (PCR) method for quantification of *C. perfringens* (138) and for the detection of the alpha-toxin gene within *C. perfringens* isolated from the gastrointestinal tract of chickens has also been reported (71).

Differential Diagnosis

Diseases that must be differentiated from NE are ulcerative enteritis (UE), *Eimeria brunetti* or *Eimeria maxima* infection. Ulcerative enteritis is caused by *C. colinum* (see "Ulcerative Enteritis"). Characteristic gross lesions are multiple areas of focal necrosis and ulceration in the distal small intestine and ceca and areas of necrosis in the liver. As described previously, lesions of NE usually are confined to jejunum and ileum with little or no involvement of ceca. These distinguishing characteristics should allow differentiation of NE and UE. Isolation and identification of the causative agent will confirm the diagnosis. *E. brunetti* infection (see "Coccidiosis," in Chapter 28) causes gross lesions similar to those produced by *C. perfringens*; however, microscopic examination of fecal smears, impressions, or intestinal sections should demonstrate the presence or absence of coccidia. Finally, NE and coccidiosis often occur concurrently in a flock. Therefore, demonstration of one or both agents is necessary for an accurate diagnosis.

Intervention Strategies

Because the sub-clinical and mild forms of NE are so detrimental to efficient performance, management of the disease should focus on controlling those factors which most put flocks at risk: coccidiosis, dietary factors and litter condition. In cases of clinical NE, early detection and treatment are necessary to prevent "seeding down" of the environment.

Management Procedures

Where a high environmental challenge exists (high soil spore counts) and repeat outbreaks occur, the addition of NaCl to poultry house dirt floors (60–75#/1000 ft^2) following a thorough clean out may prevent recurrence of the disease. Others have reported that placing birds on acidified litter aids in reducing horizontal spread of *C. perfringens* in chickens (49). Cleaning and disinfection of live haul containers with either 5% sodium hypochlorite solution or 0.4% quaternary ammonia solution have been shown to significantly reduce *C. perfringens* recovery (88).

Vaccination

Active and passive immunity through vaccination against *C. perfringens* and its toxins appears to offer good protection against infection. Immunization of chickens with a virulent strain of *C. perfringens* followed by an antibiotic treatment protected birds against a challenge infection with *C. perfringens*. Protection was also induced through oral vaccination with a live alpha-toxin-deficient isolate of *C. perfringens* (125).

The C-terminal of the alpha-toxin protein derived from avian *C. perfringens* isolates has been shown to be highly conserved (114). Antibody produced against this highly conserved region of the protein has been shown to provide protection in other species (137), and it is speculated that it may have the same effect in poultry (46, 114). Others have shown that immunization of broiler breeders with alpha-toxin vaccines produces an antibody response that appears to be protective in progeny against sub-clinical *C. perfringens*-associated NE and hepatitis (82).

Vaccination against coccidiosis may indirectly prevent the development of NE since the presence of *Eimeria* species is a predisposing factor (131, 136).

Competitive Exclusion, Probiotics, and Prebiotics

Experimentally, competitive exclusion treatments have been shown to be effective in lowering numbers of *C. perfringens* in the intestinal tract (75) while also reducing the number of gross lesions, mortality and performance losses associated with NE infections (30, 41, 58, 59, 60). The addition of mannan-oligosaccharide in addition to administration of a lactic acid-producing bacterial culture also was effective in reducing NE-associated mortality and its sub-clinical effects on performance (58). Others have shown that administration of probiotics, such as *Lactobacillus acidophilus* and *Streptococcus faecium*, reduce the severity of NE (47). *Bacillus subtilis* strains have been shown to produce bacteriocins which inhibit the growth of *C. perfringens in vitro* (124). Dietary lactose supplementation has also been shown to reduce cecal carriage of *C. perfringens* in chickens (123).

Under field conditions, treatment with a commercial competitive exclusion product was associated with improvement in intestinal health, delayed proliferation of *C. perfringens* within the intestine and delayed appearance of *C. perfringens*-associated lesions (69).

Other compounds have been investigated as potential preventive or therapeutic agents for NE. The administration of ß-mannanase to experimentally infected chicks significantly reduced the severity of the NE infection (63). Essential oil blends derived from plants have also been shown to control *C. perfringens* colonization and proliferation in the intestine of chickens (91).

Antibiotics and Anticoccidials

A number of antibiotics placed in the feed have been shown to reduce the numbers of *C. perfringens* shed in feces of chickens (119, 121, 122). These include virginiamycin, tylosin, penicillin, ampicillin, bacitracin, and furazolidone. *In vitro* antimicrobial susceptibility has also been documented for *C. perfringens* isolates from commercial turkeys and broilers (132).

Outbreaks of NE have been effectively treated with the administration of lincomycin (55, 56), bacitracin (105), oxytetracycline (7), penicillin (73, 78), and tylosin tartrate (73) in the water.

Bacitracin (104, 133), lincomycin (86), virginiamycin (35, 51), penicillin (95), avoparcin (68, 93, 104), nitrovin (94) and tylosin (19, 24) have been shown to be effective in preventing and controlling NE when placed in the feed. One survey of field isolates from clinical NE outbreaks, however, found resistance to bacitracin and lincomycin but sensitivity to virginiamycin and penicillin (31). In studies done following the removal of growth enhancing antibiotics in Scandinavia, isolates were found to be sensitive to the antibiotics ampicillin, avilamycin, erythromycin, tylosin, vancomycin, bacitracin and virginiamycin and the ionophore anticoccidials, lasalocid, maduramycin, monensin, narasin and salinomycin (64, 85). Monensin has been shown to provide a protective effect against infection through altering the microbial ecology of the ileum; reducing ileal lactobacilli populations and increasing *C. lituseburense* and *C. irregularis* populations (84). In this case it was suggested that the non-*C. perfringens* clostridia actually competitively exclude *C. perfringens*.

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Botulism

John E. Dohms

Introduction

Botulism is an intoxication caused by exotoxins of *Clostridium botulinum.* Synonyms are “limberneck” and “Western duck sickness.” Free-ranging and confinement-reared poultry and feral birds can be affected. Most avian cases are caused by *C. botulinum* type C, although outbreaks due to other toxin types have been described (5, 42).

The public health significance of avian type C outbreaks is considered minimal (5, 33). Four human type C botulism intoxi-

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cations have been reported but are not well documented (29, 33). No human cases of type C botulism have been associated with concurrent outbreaks of avian botulism (33, 63). Nonhuman primates, however, have succumbed to type C botulism after toxin inoculation (65) and captive monkeys died after eating chicken contaminated with type C toxin (60).

History

Botulism was first reported in chickens in 1917 (13). Both chickens and humans developed the disease after ingestion of home-canned vegetables. Western duck sickness, first recognized in the United States in the early 1900s, was later found to be caused by *C. botulinum* type C toxin (23, 34). Botulism in chickens following ingestion of *Lucilia* fly larvae was reported in 1923. The first *C. botulinum* type C strains also were isolated from these invertebrates (4). For additional historic information, see (9, 15, 40, 55).

Incidence and Distribution

The disease has affected poultry and waterfowl worldwide (33). Although many early cases occurred in free-ranging poultry, and modern methods of poultry husbandry were thought to have reduced the incidence of botulism by preventing access to toxin-contaminated food, severe cases have been reported in confined broiler flocks (5, 15, 28, 49, 50, 52, 53, 61). All types of wild birds and both avian and mammalian predators and scavengers have been affected during type C botulism outbreaks in wild birds (33). In these outbreaks, ducks have been most often affected (55). Botulism has been reported in pheasants reared on game farms (55). Botulism in ducks, broiler chickens, and pheasants occurs more frequently and with greater severity during warmer months. However, outbreaks in broiler chickens also have been reported in winter (17, 51).

Etiology

C. botulinum is a gram-positive, spore-forming bacterium capable of elaborating potent exotoxins under appropriate environmental conditions (42).The species consists of a diverse group of anaerobic bacteria including 4 cultural (1–IV) and 8 antigenically different toxigenic groupings (A, B, C alpha, C beta, D, E, F and G). Human disease has been associated mainly with types A, B, E, and F, while A, C, and E have caused disease in birds (62). Cases of botulism in chickens, ducks, pheasants, and turkeys in natural or commercial settings have been caused primarily by type C toxigenic group (15, 55, 61).

Morphology and Staining

The gram-positive cells of *C. botulinum* type C measure 4–6 × 1.0 um, often occurring singly or in short chains. The vegetative cell is motile. Subterminal or occasional terminal endospores are present in aging cultures (42). A cell-wall lysin is responsible for rapid autolysis of the organism and causes gram-variable staining in older cultures. Toxin is released during autolysis (7). Type C spores are more easily heat inactivated than type A and B spores (42) but are more resistant to heat than type E spores (58). The time required to cause a 10-fold reduction in spore viability at 101°C (D value) was 2.44 min for a terrestrial type C strain (58).

Culture group III contains nonproteolytic or weakly proteolytic type C and D toxigenic types (30, 63). *C botulinum* requires available water content (a_w) of 0.92 for growth and toxin production (52). The type C toxigenic group is further subdivided into C alpha and C beta subtypes based on their toxigenic properties (42).

Toxins

Botulism exotoxins are among the most potent toxins known (39). Type C toxin is produced under anaerobic conditions at temperatures between 10° and 47°C with optimal toxin production between 35 and 37°C (42). The neurotoxins action occurs at the peripheral cholinergic nerve terminus. The neurons action occurs at the peripheral cholinergic nerve terminus.

Type C alpha cultures produce three toxins; C1, C2, and small amounts of type D toxin (20). C1 and D toxin production is mediated by bacteriophage. Type C strains, cured of their prophage, can be converted to type D organisms by infection with phage purified from type D strains. The reciprocal is also true (20). Type C beta strains, lacking bacteriophage encoding C1 and D toxins, produce only C2 toxin; genes encoding C2 toxin are not phage associated (20). Because of the interconverabilty of C and C beta strains, the relevance of C alpha and C beta toxigenic grouping has been questioned (20).

C1 and D, together with A, B, E, and F toxins, are synthesized as single nontoxic progenitor polypeptides that are later cleaved by proteases to produce 150-kD dichain neurotoxins (44, 59, 64). The progenitor type C toxin is associated with a nontoxic nonhemagglutinin and hemaggluting proteins which increase resistance to acid and protease degradation. However, at the mildly alkaline pH of the small intestine, a protease-sensitive loop is cleaved, producing a 100-kD heavy chain and a 50-kD light (L) chain, held together by an interchain disulfide bond. The active domains include H_N, a membrane translocation domain, H_C, a neurospecific binding domain, and L, a Zn^{+2} -dependent metalloproteinase (44). After binding to a specific receptor in the presynaptic membrane, the molecule is captured within an endocytic vesicle and acidified by a vacuolar ATPase proton pump which permits translocation into the cytosol. Reduction inside the cytosol produces the active metalloproteininase specific for two components of the presynaptic neuromuscular junction, syntaxin and SNAP-25. The outcome is persistent loss of acetylcholine release, and because acetylcholine receptors are not stimulated, muscle paralysis occurs (44).

Binary C2 toxin, although not neurotoxic, requires trypsin activation causing increased membrane permeability in a variety of cultured tissues (46, 59). The active toxin contains 2 distinct proteins, a binding and translocation protein (C2II) and an actin-ADP-ribosylating enzyme (C2I). The C2I enzyme ADP-ribosylates G actin at Arg-177, which inhibits actin polymerization and actin ATPase activity and converts actin into a capping protein that binds to actin filaments and prevents fast polymerization (1, 21, 66). It also complexes with gelsolin, altering its in-

teraction with actin (6). The C2 toxin is cytolytic in cultured cells and causes changes in the actin cytoskeleton, depolymerization of actin filaments, and cell rounding (6). Toxicity requires C2II attachment to asparagines-linked complex carbohydrate receptors after the trypsin dependent cleavage of an N terminal 20-kDa fragment (46). The trypsinized component forms heptomers that produce channels in artificial membranes. The complex is endocytosed, and the C2I enzyme translocates into the cytosol disrupting actin. Nucleic acid sequence of C2 toxin shows sequence similarity with other actin-ADP-ribosylating toxins from other *Clostridium* and *Bacillus* species (6). *C. botulinum* C2 toxin shows homology to the *Bacillus anthracis* protective antigen, which translocates edema factor, adenyl cyclase, and lethal factor into the cytosol (6).

Ducks and geese inoculated intravenously with C2 toxin showed cardiopulmonary symptoms (32, 46). In mice, C2 toxin has enterotoxic properties (46). The role of C2 toxin in natural botulism outbreaks is presently unclear.

Chickens, turkeys, pheasants, and peafowl are susceptible to types A, B, C, and E but not D or F toxin (25). Chickens are most sensitive to types A and E given intravenously but relatively resistant to type C1 intoxication (16, 25, 43, 52, 53). In contrast, ducks and pheasants are more susceptible to C1 toxin (25, 27). Compared with other toxins, C1 and C2 are more readily absorbed by chickens when given orally (25). As broiler chickens age, they become less susceptible to C1 toxin. At hatching the chicken lethal dose-50% (LD_{50}) was $10^{3.0}$ mouse-LD_{50} per kg body weight compared with $10^{6.3}$ mouse-LD_{50} per kg body weight at 8 wk of age (16).



22.5. Botulism in chickens showing paralysis of wing and lower eyelid, difficult breathing caused by partial paralysis of respiratory muscles, and ruffled hackle feathers.

Pathogenesis and Epidemiology

Natural and Experimental Hosts

Type C botulism has occurred in many species of birds including chickens, turkeys, ducks, pheasants, and ostriches (2). In wildlife outbreaks, 117 avian species in 22 families have been affected (32). Outbreaks in aviaries have occurred (61, 63). Mammalian species affected by type C toxin include mink, ferrets, cattle, pigs, dogs, horses, and a variety of zoo mammals (42). Fish succumbed to type C botulism during outbreaks on fish farms (63). Type C botulism in ruminants fed poultry manure has caused serious economic loss (19). Laboratory rodents are fully susceptible to type C toxin; mice are useful in the bioassay for toxin detection and typing.

In a study of 27 outbreaks in broiler chickens, ages ranged from 2 to 8 wk with a mean of 6.2 ± 1.7 wk (17). Outbreaks in older broiler chickens have been reported (5). Paradoxically, at these ages broiler chickens are relatively resistant to C1 toxin (16).

Incubation Period

Experimental subcutaneous, intravenous or oral inoculation of type C toxin in chickens and ducks produced clinical signs identical to those observed in field outbreaks. Morbidity and mortality were dose related. With high levels of toxin, disease appears within hours. With low toxin doses, onset of paralysis occurs within 1–2 days (16, 25, 27, 31).

Transmission

C. botulinum type C is distributed worldwide wherever large populations of wild and domestic birds are found. Type C organisms readily grow in the gastrointestinal tract of birds and are considered obligate parasites (63). Type C spores are commonly found in and around poultry and pheasant farms (17, 38, 61, 62). Presence of organisms in the gastrointestinal tract of wild and domestic birds and resistance of spores to inactivation favor spread of this organism (17, 33).

Signs

Clinical signs of botulism in chickens, turkeys, pheasants, and ducks are similar (9, 15, 31, 55).In chickens, flaccid paralysis of legs, wings, neck, and eyelids are predominant features of the disease. Paralytic signs progress cranially from the legs to include wings, neck, and eyelids. Initially, affected birds are found sitting and are reluctant to move. If coaxed to walk, they appear lame. Wings droop when paralyzed. Limberneck, the original and common name for botulism, precisely describes the paralysis of the neck (Fig. 22.5). Because of eyelid paralysis, birds appear comatose and may seem dead. Gasping has been reported when birds are handled. Death results from cardiac and respiratory failure (63).

Affected chickens have ruffled feathers, which may fall out with handling. Quivering of certain feather tracts has been ob-

served. Broiler chickens showing signs of botulism may have diarrhea with excess urates in the loose droppings.

Morbidity and Mortality

Morbidity and mortality are related to the amount of acquired toxin. Low levels of intoxication produce little mortality and morbidity, which can confuse diagnosis. In severe cases, up to 40% mortality has been observed in broiler flocks (15, 51).

Western duck sickness is one of the most devastating diseases of waterfowl. Mortality, although difficult to estimate in wild birds, was reportedly greater than 100,000 birds on separate occasions (9, 33). Such losses have major impact on wildlife populations (33). In other cases, outbreaks in small lakes have been limited to the relatively few waterfowl in these habitats (3, 61). Mortality of pheasants reared on game farms has been as high as 40,000 birds (55).

Pathology

Birds with type C botulism lack gross or microscopic lesions. Occasionally, maggots or feathers can be found in the crop or affected birds.

Pathogenesis

Type C botulism can be caused by ingestion of preformed toxin. Because the organism is widely distributed in the gut, dead birds provide conditions for *C. botulinum* growth and toxin production. Greater than 2000 minimum lethal doses (MLD) of type C toxin per gram of carcass tissue have been found (5). Birds scavenging such carcasses can readily obtain enough toxin to become affected. Fly-blown carcasses may have maggots containing varying levels of botulism toxin. Maggots have been found to contain from 10^4–10^5 MLD of toxin (61). Maggots are readily devoured by chickens, pheasants, or ducks, which can lead to explosive botulism outbreaks. In aquatic environments, small crustaceans and insect larvae may contain *C. botulinum* in their gut. If large numbers die due to oxygen depletion, toxin can be produced within these invertebrates. Ingestion of toxin laden invertebrates has been proposed as the cause of type C botulism in ducks (55, 68). Lakes with shallow sloping banks that experience dramatic fluctuations in water level are most commonly associated with botulism outbreaks (33, 68).

Botulism caused by A and E occurs rarely and generally has been associated with consumption of spoiled human food products fed to backyard chicken flocks (40). Botulism in sea gulls, loons, and grebes was caused by eating dead or dying fish contaminated with type E toxin (42). A case of type A botulism in broiler chickens was due to a contaminated feed source (12).

The pathogenesis of botulism was once exclusively thought to be due to ingestion of preformed toxin. There is growing evidence that *C. botulinum* type C elaborates toxin *in vivo* to cause disease (61). The term *toxico-infection,* originally used by Russian researchers, was adapted to describe this form of the disease in broiler chickens (51, 63). In two cases of type C botulism in broiler chickens, carcasses were implicated as the source (5, 28). In the majority of broiler chicken outbreaks however despite comprehensive searches, no toxin sources have been identified (17, 26, 49, 57, 61). The disease pattern in many of these outbreaks was inconsistent with food or water as toxin sources. Dead carcasses could not account for intoxications.

Type C botulism was reproduced in leghorn chickens and pheasants fed botulinal spores. Chickens and pheasants with their ceca ligated had a lower incidence of disease following spore challenge (38, 43) suggesting the cecum as the site of toxin production. In pheasants, the crop supported toxin production (14). Attempts to reproduce the toxicoinfectious form of botulism in broiler chickens have in the past been unsuccessful (15, 38). Toxin has been produced in the cecum of broiler chickens, but not at levels sufficient to kill the host (38). Chickens treated with the immunosuppressive drug cyclophosphamide before *C. botulinum* type C spore inoculation showed cecal colonization and reproduction of clinical botulism, perhaps suggesting that stress or viral induced immunosuppression might predispose chickens to the disease (48). An environmental, bacterial, phage, and/or host interaction may be required for toxicoinfections botulism to occur in broilers.

Immunity

Because the toxigenic dose is lower than the immunogenic dose, chickens and ducks recovering from botulism do not develop immunity (8, 25). However, carrion-eating crows and turkey vultures possessed antibodies to botulinal toxin (39, 47). This may partly explain why vultures were resistant to experimental inoculations of toxin (35).

Diagnosis

The differential diagnosis of botulism is based on clinical signs and lack of gross or microscopic lesions. Definitive diagnosis requires detection of toxin in serum, crop, or gastrointestinal washings from morbid birds (15, 63).

Serum is the preferred diagnostic sample. Because *C. botulinum* is found in the gut of normal chickens, toxin can be produced in decaying body tissues. Therefore, finding toxin in tissues of dead birds does not confirm botulism.

The mouse bioassay is a sensitive and reliable method for confirming heat-labile toxin in serum (15). Groups of mice are inoculated with suspect serum samples. Other mice receive samples inoculated with suspect serum samples treated with type-specific antiserum. If toxin is present in the sample, signs and death of mice given untreated samples usually occur within 48 hr. Mice inoculated with antitoxin will be protected. Other *in vitro* methods of detecting toxin have been reviewed (45). An antigen-capture ELISA assay for *C. botulinum* type C toxin was able to detect 0.25ng/ml toxin compare to 0.12ng/ml detection using the mouse bioassay. However, using larger sample volumes in ELISA proved to be as sensitive to the mouse test due to the concentrating effect of the capture antibody (54).

In waterfowl and some poultry outbreaks, toxin levels in blood may be too low to produce disease in mice. Concentration of serum or repeated inoculation of mice with suspect serum may be required to demonstrate toxin in these cases (27). More recently a sensitive and specific immunoassay for type C and D was developed alleviating the use of the mouse bioassay (22).

In advanced stages of the disease, clinical signs are obvious; during mild intoxications, only leg paralysis may be observed. The mild form of the disease must be differentiated from Marek's disease, drug and chemical toxicity, or appendicular skeletal problems. In these cases, the mouse bioassay is particularly helpful in diagnosis. Eyelid paralysis is a key sign differentiating botulism from other conditions. Botulism in waterfowl must be differentiated from fowl cholera and chemical toxicities. Lead poisoning of waterfowl is commonly confused with botulism (55, 63).

Isolation of *C. botulinum* requires anaerobic culturing and is of little help in diagnosis (30). The organism is widely distributed in gut, liver, and spleen of clinically normal chickens (16). Detection of the organism, however, in feed or environmental samples may prove useful in epidemiologic studies. The organism can be demonstrated in samples inoculated into cooked-meat medium and inoculated anaerobically at 30°C (11). After 3–5 days incubation, toxin can be detected using mouse bioassay with specific typing antitoxins. Other modifications of this procedure are available (30, 63). The organism can be detected using the fluorescent antibody technique (41).

Treatment

Many sick birds, if isolated and provided with water and feed, will recover. Treatment of large numbers of morbid birds, however, is difficult, and various protocols have been used but not verified experimentally. The success of these treatments is hard to establish because of the difficulty in experimentally reproducing toxicoinfectious botulism. The patterns of disease in untreated broiler houses can rise and fall during a given outbreak (17). Therefore, it is difficult to know whether a particular treatment is effective, or, if by chance, treatment precedes a drop in mortality that would have occurred anyway. However, several treatments have been reported to be of benefit. Treatment of affected broiler flocks with sodium selenite and vitamins A, D_3, and E reduced mortality (57). Antibiotics including bacitracin (100 g/ton in feed), streptomycin (1g/L in water), or periodic chlorotetracycline treatments also reduced mortality (55). Penicillin was ineffective in controlling one outbreak (49) but has been found efficacious in other affected flocks (51). *In vitro* susceptibility of *C. botulinum* to 13 antibiotics include tetracycline, metronidazole, erythromycin, penicillin, rifampin, chloramphenicol, clindamycin, cephalothin, cefoxitin, and vancomycin (56).

Inoculation with specific antitoxin neutralizes only free and extracellular bound toxin and might be considered for treating valuable birds in zoologic collections. Ostriches showing clinical signs of botulism responded favorably within 24 hr after treatment with type C antitoxin (2). This is impractical in commercial poultry, duck, or pheasant outbreaks.

Prevention and Control

Management practices should emphasize removal of potential sources of the organism and its toxin from the environment. Prompt disposal of dead birds and culling of sick birds is very important in prevention and control. In problem areas, removal of contaminated litter and thorough disinfection using calcium hypochlorite, iodophor or formalin disinfectants may help reduce spore numbers in the environment (56). In houses with dirt floors, complete destruction of these spore formers is difficult. Disinfection of areas around poultry houses has been recommended (56). Spores may be located in soil outside of the poultry facility and can be transported back into houses. Fly control may be another means of reducing the risk of toxic maggots in the environment. During outbreaks, it has been suggested that feeding lower energy diets reduces mortality caused by toxicoinfectious botulism (57). Two cases of type C botulism were reported in commercial broilers and were associated with elevated intake of iron from water and feed sources, iron having been shown to promote the proliferation of many enteric bacteria including *C. botulinum* (50). In the first case, the outbreak took place in a house where water was drawn from a well with elevated iron levels (1.35 ppm), while an adjacent house with a different well source (iron level 0.66 ppm) was botulism-free. In the second case type C botulism outbreaks occurred on farms geographically separated but operated by the same company. Elevated iron was found in the drinking water on one farm while on the second affected farm, broiler feed contained 34,000 ppm iron. To confirm the relationship of iron and botulism, experimental reproduction of toxicoinfectious type C botulism must be demonstrated. Acidification of drinking water with citric acid has been suggested as a method of lowering gut pH which promotes more favorable growth of normal flora, inhibits *C. botulinum* growth and acts as a heavy metal chelator (10, 11, 24, 36, 50, 67).

Immunization

Active immunization with inactivated bacterin-toxoids has been successfully used in pheasant operations (37). Similarly formulated toxoids protect chickens and ducks from experimental botulism (8, 18). Vaccination of large numbers of broiler chickens is costly and vaccination of wildfowl is not practical.

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Gangrenous Dermatitis

Kenneth Opengart

Introduction

Definition and Synonyms

Gangrenous dermatitis (GD) is a disease of chickens and turkeys caused by *Clostridium perfringens* type A*, C. septicum or Staphylococcus aureus*. The disease is characterized by a sudden onset of acute mortality. The primary lesion in affected birds is necrosis of the skin and subcutaneous tissue, usually involving the breast, abdomen, wing or thigh. Gangrenous dermatitis has also been referred to as necrotic dermatitis, gangrenous cellulitis, gangrenous dermatomyositis, avian malignant edema, gas edema disease, wing rot, and, in some instances, blue wing disease—a component of CIAV infection (7, 44, 53).

Economic Significance

The economic impact of GD is primarily related to the degree of GD-associated mortality that occurs within a flock. Gangrenous dermatitis usually affects mature broilers and turkeys and the economic losses due to the disease are, therefore, associated with any lost investment in production costs (chick/poult cost and feed) and the loss of income related to the reduction in marketable pounds.

Public Health Significance

Gangrenous dermatitis is a disease characterized by acute mortality, lesions which are localized rather than systemic and a relatively short incubation period. Most affected birds succumb quickly to infection and do not make it to processing age. Therefore, the public health significance of GD is thought to be minimal.

History

Severe necrosis of muscle and subcutaneous tissue following intramuscular inoculation of *Clostridium welchii* (*C. perfringens*) isolated from heart blood and liver of 2 chickens was first described in 1930 (41). The following year, *C. perfringens, C. sep-*

ticum, and *C. novyi* were isolated from a diagnostic case in which chickens were dying of wound infections following the collection of blood samples for pullorum testing (5). Death of turkey breeder hens from wound infections occurring during mating caused by *C. perfringens*, *C. septicum*, and *C. sordellii* was reported in 1939 (16). Since that time GD has been reported in chickens and turkeys throughout the world (2, 4, 6, 8, 9, 10, 11, 18, 19, 20, 27, 28, 29, 34, 38, 46, 48, 50).

Etiology

Classification

Causes of GD are *C. septicum* (18, 19, 27, 28, 34, 49), *C. perfringens* type A (4, 11, 54), and *S. aureus* (6, 9, 20, 34), either singly or in combination (19, 29, 34, 48), with combined infections generally being more severe. Frequency, severity of lesion production and resulting mortality depend upon the specific bacterial strains involved in the infection and their ability to produce toxins (55).

Morphology and Growth Requirements

Isolation and identification of *S. aureus* and *C. perfringens* have been described elsewhere (see Chapter 23, "Staphylococcosis" and "Necrotic Enteritis" in this chapter). Culture for *C. septicum* should be carried out anaerobically on blood agar plates containing 2.5% agar, which will reduce the swarming of *C. septicum* over the plate surface (17). Incubation is for 1–2 days at 37°C. Positive identification of the organism is made by inoculation of differential media (1).

Biochemical Properties

The biochemical properties of *S. aureus* and *C. perfringens* are described elsewhere (see Chapter 23, "Staphylococcosis" and "Necrotic Enteritis" in this chapter). *C. septicum* ferments glucose, maltose, lactose, and salicin but not sucrose or mannitol. Principal products of fermentation are acetic and butyric acids. Gelatin is hydrolyzed, milk is not digested and indole is not produced. Growth on egg yolk agar demonstrates an absence of lecithinase and lipase production. Clostridial spores are oval and located subterminally.

Pathobiology and Epidemiology

Incidence, Distribution, Hosts

While natural outbreaks of GD have been described in chickens from 17 days to 20 weeks of age, most have been reported in 4- to 8-week-old broiler chickens (4, 6, 9, 18, 19, 27, 28, 29, 34, 48). The disease has also been reported in 6- to 20-week-old commercial layers (19, 48), 20-week-old broiler breeders (20), and chickens following caponization (54). Subcutaneous emphysema in chickens, from which *C. perfringens* was isolated, was described in Israel in 1950 (46). Since 1963, there have been reports of gangrenous dermatitis from various parts of the world including the United States (19, 48), the United Kingdom (18), Germany (28, 34), Belgium (20), Argentina (4), New Zealand (38), Egypt (2) and India (10, 11, 50).

In turkey breeder hens, cellulitis and mortality associated with clostridia and gram-positive cocci infections have been reported (16). *Clostridium perfringens* type A has also been isolated from the tail and ventrum of market age commercial turkey flocks experiencing cellulitis and excessive mortality (8).

Experimental reproduction of GD in chickens (19, 27, 28, 34, 48) and turkeys (48) following intramuscular or subcutaneous inoculation of *C. septicum*, *C. perfringens* type A, or *S. aureus* has been reported to cause mortality and lesions similar to those that occur in naturally occurring outbreaks. Intramuscular inoculation with *C. septicum* isolated from chickens caused death in turkeys within 24 hours with circumscribed lesions at the inoculation site (48).

Transmission, Carriers, and Vectors

Clostridia are present in soil, feces, dust, contaminated litter or feed and intestinal contents (1, 33). Staphylococci are ubiquitous and common inhabitants of skin and mucous membranes of poultry and areas where poultry are hatched, reared, and processed (see Chapter 23, "Staphylococcosis").

Clinical Signs

Clinical signs in naturally occurring outbreaks of GD include varying degrees of depression, incoordination, inappetence, leg weakness, and ataxia (18, 19, 27, 28, 48). Because the period of illness is short, usually less than 24 hours, birds are often well-fleshed and hydrated. Mortality occurs acutely and can range from 1 to 60% (18).

Pathology

Gross

Gross lesions consist of dark reddish-purple, weepy areas of the skin, usually devoid of feathers. Affected areas usually include wings, breast, abdomen, or legs (see Figs. 22.4E, F) (11, 18, 19, 27, 28, 48). Extensive blood-tinged edema, with or without gas (emphysema), is present beneath the affected skin (Fig. 22.4G). Underlying musculature is discolored gray or tan and may contain edema and gas between muscle bundles. In some cases, emphysema and serosanguineous fluid are present in subcutaneous tissue, but there is no loss of integrity to the overlying skin (29). Most cases report no internal lesions; however, discrete white foci (necrosis) in the liver (9, 48) and small flaccid bursae of Fabricius (9, 29), the latter presumably due to IBD virus infection, have been reported in affected birds. In turkeys with cellulitis of the tail, edema and vesicle-like lesions were present laterally and ventrally around the tail. Tail feather shafts were soft, blood-filled, and were often broken (8).

Microscopic

Microscopic changes are characterized by edema and emphysema (Fig. 22.4H) with numerous large, basophilic bacilli or small cocci within subcutaneous tissues (9, 48). Severe congestion, hemorrhage and necrosis of underlying skeletal muscle are often present. Liver, if affected, contains small, randomly scattered, discrete areas of coagulation necrosis with intralesional bacteria. Bursal changes, in cases suspected to have concurrent

IBD, are characterized by extensive follicular necrosis and atrophy (9, 29).

Pathogenesis of the Infectious Process

Contributing Factors

In many instances, GD is believed to occur as a sequela to other diseases which produce immunosuppressive effects such as infectious bursal disease (IBD) virus, chicken infectious anemia virus (CIAV) (7, 23, 44, 53), reticuloendotheliosis virus (31), and avian adenovirus infections, including inclusion body hepatitis virus (9, 18, 29, 36, 40, 47). In addition, some outbreaks of GD have been reported to be breeder flock-associated (i.e., progeny from specific breeder flocks consistently develop GD) (21). Lack of antibody to IBD virus in broiler breeders correlates with increased susceptibility of their progeny to dermatitis (47).

Lesions characteristic of chicken infectious anemia virus-induced GD (blue wing disease) include intracutaneous, subcutaneous, and intramuscular hemorrhages and edema as well as atrophy of thymus, spleen, and bursa of Fabricius (14). Numerous avian reoviruses and CIAV have been isolated from chickens affected with blue wing disease (7, 14), and GD has been reproduced by dual infection with CIAV and a reovirus (15). Gangrenous dermatitis often occurs secondarily to the hemorrhages associated with blue wing disease. A compromised immune system is thought to be the underlying predisposing factor associated with GD in these birds (13, 25).

Environmental factors (high litter moisture due to poor litter quality, poor drinker management or poor ventilation) may also predispose flocks to GD, especially when they occur in conjunction with challenges from immunosuppressive viruses. Poor farm management, especially failing to remove dead birds from an area in a timely manner, may also predispose a flock to GD. Factors that lead to increased scratches, such as overcrowding, feed outages, meal time feeding, and bird migration in tunnel ventilated houses, can increase GD incidence (56). The incidence of GD has also been observed to be related to the season of the year with peak occurrence in the spring. In the absence of other predisposing factors such as immunosuppressive infectious agents and management factors, GD tends to be associated with certain strains or breeds, males more often than females, and flocks which perform above production standards. Affected farms tend to have repeat outbreaks.

Diagnosis

A diagnosis of GD can be confirmed with the presence of typical gross and microscopic lesions and the isolation of the causative agent(s). In field cases of GD, staphylococci and clostridia can be isolated from exudates of skin and subcutaneous tissue or underlying muscle (9, 11, 19, 28, 48). Identification of the causative agent(s) can be done as described under "Etiology." As mentioned previously, occurrence of GD often can be preceded by other infectious agents affecting the immune function of the bird. Diagnosis of an underlying etiology is often necessary to fully understand the complexity of GD infection.

Differential Diagnosis

A variety of skin conditions must be differentiated from GD. Contact or ulcerative dermatitis ("breast burn") of broiler chickens (24, 37) and plantar pododermatitis of turkeys (37) are conditions characterized by erosions and ulcers accompanied by acute inflammatory changes over the breast, hock, and plantar surface of the feet. A strong correlation between wet or poor litter and these conditions is present (37, 39). Infectious or inflammatory process (IP), a condition usually caused by *E. coli* in market age broilers involving the subcutaneous tissues of the abdomen, thigh, and leg, can also cause the dermis to appear reddened and edematous (22). However, this condition never has emphysematous lesions or mortality associated with it and is generally only a problem observed at the processing plant. Scabby hip dermatitis is a syndrome of broilers that, like contact dermatitis, is a nonspecific dermatitis with ulceration and secondary bacterial infection (26). Lesions have been correlated with high stocking densities which may cause feather breakage and scratches of the lumbar and sacral regions allowing entrance of bacteria into the dermis (26, 45). To differentiate GD from these conditions, demonstration of poor environmental conditions or overcrowding and the lack of an association with a primary immunosuppressive infectious agent must be demonstrated.

Squamous cell carcinoma (now known as avian keratocanthoma) which can lead to ulceration and infection of the epidermis may be difficult to differentiate from GD (52). Histopathology of the affected area may be necessary to differentiate the two conditions.

Additionally, a number of nutritional deficiencies and genetically slow-feathering male chickens may predispose birds to GD (12).

Dermatitis caused by mycotic agents *Rhodotorula mucilaginosa* (3), *R. glutins* (42), *Candida albicans* (35), and *Aspergillus fumigatus* (57) can be differentiated from GD by the demonstration of fungal elements in impression smears or tissue sections and by isolating and identifying the agent. Vesicular lesions involving the wattles, comb, shanks, and feet have been described in chickens (30, 43, 51) and have been suspected or proven to be due to the ingestion of the fungus *Cladosporium herbarum*, producing an ergot-like disease, or *Ammi visnaga* seeds, which lead to photosensitization. These lesions invariably occur only on unfeathered areas of the skin and should be differentiated easily.

Intervention Strategies

Management Procedures

Prevention has been accomplished by managing the placement of susceptible breeds on at-risk farms. Additionally, total clean out of farms, followed by thorough cleaning and disinfection of the house and floor, has helped resolve farms with historical problems. In these cases, large amounts of water mixed with a phenolic disinfectant (1500 gallons per 20,000 ft^2) have been used to achieve a saturation depth of 3–4 inches of the dirt floor pad. Treating the floor with salt at 60–100 pounds per 1,000 ft^2 prior to placement of bedding material also has decreased the incidence of GD on problem farms. Generally, management procedures to improve litter condition, reduce litter moisture, acidify litter pH, reduce bacterial levels in the environment and mini-

mize trauma are useful adjuncts to other prevention and control methods.

Vaccination

Administration of a mixed clostridial bacterin at 1 day of age has been shown to reduce losses in flocks due to GD (21). Similar results have been reported in 5-week-old chickens vaccinated with a mixed *E. coli*, *S. aureus* and *C. perfringens* bacterin following bacterial challenge with live cultures of the same organisms (32).

Treatment

Outbreaks of GD have been treated effectively with the administration of chlortetracycline (27), oxytetracycline (48), erythromycin (48), penicillin (9, 11, 29), or copper sulfate (2) in the water and chlortetracycline (28, 48) or furoxone (28) in the feed. However, in many instances, antibiotics used for control have proved to be of limited use (18, 20, 21, 34). Failure of antibiotic treatment can often be explained by the fact that an underlying immunosuppressive viral infection is usually a predisposing factor for GD. The birds, therefore, are unable to completely clear the bacterial infection even with the presence of an antibiotic. For this reason, modification of vaccine programs directed at immunosuppressive agents like IBD and CIAV may sometimes be used to combat widespread GD problems.

Water acidification, with citric and proprionic acid, have been used to reduce, but not eliminate, mortality in flocks where the rate of mortality does not dictate the use of an antibiotic or where antibiotics are no longer effective.

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Chapter 23

Other Bacterial Diseases

Introduction

H. John Barnes

Collectively, bacterial diseases continue to be a significant cause of economic loss in the poultry industry. Those that are common, widespread, or of major public health significance are covered elsewhere in individual chapters. Diseases caused by bacteria that are sporadic or of limited occurrence are reviewed in this chapter. Significant losses from these diseases still may occur in an affected flock, or rarely an integrated company, but their impact on the entire poultry industry is not considered great. Enterococcal infections are included with streptococcal infections. Enterococci continue to increase in importance as use of growth promotants and antibiotics are being minimized (2, 8), and they have been associated with a variety of local and systemic diseases (1, 3, 5, 6, 9), while streptococcosis is rarely seen.

Bacteria that are isolated from sick or dead birds but have an unknown role, are infrequently associated with disease, or are primarily of public health significance have been grouped together in a concluding subchapter comprised of sections about each genus or disease. Readers are referred to earlier editions of *Diseases of Poultry* for information on *Bacillus anthracis* (anthrax), *Brucella, Cowdria, Coxiella, and Francisella* (tularemia). While infections of poultry and other birds with these organisms are possible, they either do not produce clinical disease or have not been reported recently. The section on turkey osteomyelitis complex has been moved to the chapter on colibacillosis to better reflect the nature of that disease and information on megabacteria, which is not a bacterium but a yeast (*Macrorhabdus*), and can be found in the chapter on fungal infections.

Improved methods of taxonomic classification based on genomics have resulted in removal of some bacteria from an existing genus and placement into a new one (e.g., *Actinobacillus salpingitidis,* avian *Pasteurella haemolytica*-like, or *P. anatis* placed into a new genus *Gallibacterium*) or creation of new genera or species for previously unnamed organisms (e.g., *Coenonia, Pelistega, Suttonella*). *Arcobacter* and *Helicobacter* are composed of bacteria that were formerly considered atypical campylobacters or campylobacter-like organisms. They have the same capacity as *Campylobacter* to cause food-borne illness in people but appear to be relatively innocuous for poultry (7). Knowledge concerning the host and geographic distribution, public health significance, disease associations, and species of helicobacters continues to expand. *Listeria* is another bacterium of considerable importance as a cause of human disease, but in poultry, it rarely causes clinical disease.

Some diseases such as beak necrosis, venereal disease of geese, and liver granulomas appear to have a bacterial cause, but because of their multifactorial nature, specific organisms have not been identified. Similarly, a microorganism consistent with a spore-forming bacterium has been seen in lesions in Muscovy ducks, but its identity remains unknown.

Bacteria can be primary pathogens, but this is infrequent compared to the roles they play as opportunists or co-pathogens. Often Koch's postulates cannot be fulfilled, even though a specific bacterium is associated with a lesion or disease. When many factors, in addition to an infectious agent, contribute to the cause of a disease, it may be impossible to duplicate the required conditions to reproduce it experimentally. In such situations, it may be possible to use Evans' postulates to document the role of a bacterium in such a disease (4).

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Staphylococcosis

Claire B. Andreasen

Introduction

Staphylococcus infections are common in poultry. These infections are mainly caused by *Staphylococcus aureus*, although other species are occasionally involved (8, 19, 102, 112). The symptoms vary with the site of entry. The most frequent sites are the bones, tendon sheaths, and joints, especially the tibiotarsal and stifle joints (Table 23.1). Staphylococcal infections occur less frequently in other locations including skin (43, 56, 100, 102, 127), sternal bursa (121), yolk sac (131), heart (13), vertebrae (17), eyelid (19), and as granulomas in the liver and lungs (7, 77, 87). Infections are usually characterized by increased heterophil counts and marked heterophilic infiltration of tendons, synovial membranes, and other affected organs (4). Staphylococcal septicemia, causing acute deaths in laying birds (14), seems to be most prevalent in hot weather and resembles fowl cholera. The route of entry, pathogenesis, and host response are not completely defined. Staphylococcal disease is usually chronic and responds poorly to antimicrobial therapy or immunization.

Economic Significance

Staphylococcal infections are a worldwide problem in chickens and turkeys and cause economic losses due to decreased weight gain, decreased egg production, and condemnation of carcasses at slaughter (88). During the processing of turkeys, a high correlation between green-discolored livers and *S. aureus* has been made and termed green-liver osteomyelitis complex (12, 20). Although *S. aureus* is the most commonly isolated pathogen in this condition, *Escherichia coli* and numerous opportunistic bacteria have also been isolated from affected turkeys (12).

Public Health Significance

In addition to being a major disease-producing organism for poultry, approximately 50% of typical and atypical *S. aureus* strains produce enterotoxins that can cause food poisoning in human beings (41, 45, 53, 99, 113). Poultry-associated food poisoning can occur due to the contamination of poultry carcasses with these enterotoxin-producing *S. aureus* strains at processing. *S. aureus* strains from processed poultry are thought to be endemic to the processing plant or from the hands of workers in the plant (1, 71, 96, 115). The literature varies as to the origin of processing plant strains with biotyping indicating the passage of human staphylococcal strains to poultry in processing plants; plasmid profiling indicates that endemic strains in the processing plant are introduced by incoming birds (35).

Methicillin-resistant *Staphylococcus aureus* (MRSA), which has emerged as an important human pathogen, may also be a concern in chicken meat (37, 73, 125). Methicillin-resistant strains of *S. aureus* are resistant to beta-lactam antibiotics including the semi-synthetic penicillins (125). Many isolates are also resistant to numerous other antibiotics including the fluoroquinolones. Although there have been no reports of transmission between poultry and humans, MRSA has uncommonly been transmitted between humans and companion animals or horses (9, 37, 75, 79, 103, 119, 120, 125, 126). The possibility of MRSA transmission to humans in foods of animal origin, including poultry, has also been proposed (73). MRSA has been reported in poultry. In Korea, where MRSA is common in humans, 421 of 1913 specimens from cattle, pigs, and chickens collected between 2001 and 2003 contained *S. aureus* (73). Three isolates from chickens, one from a suppurative area in the meat and two from joints, were MRSA. MRSA has also been isolated from raw retail chicken meat in Japan (65). In addition, it is theoretically possible for the *mecA* gene, which is responsible for methicillin resistance, to be transmitted between species of staphylococci found in animals and staphylococci found in humans (37). For this reason, methicillin-resistant species other than *S. aureus* can also be a concern in poultry. At one farm in Japan, *S. sciuri, S. epidermidis,* and *S. saprophyticus* all containing the *mecA* gene were isolated from the nares and skin of healthy chickens (61).

History

Staphylococcosis in poultry and other avian species has been recognized for more than 100 years; most early reports describe arthritis and synovitis (51, 57, 60, 78).

Etiology

Classification

The genus *Staphylococcus* contains approximately 36 species and 21 subspecies (40). It is the most important genus in the family Staphylococcaceae. The general term staphylococcus refers to the morphology of these microorganisms; in stained smears, they often resemble clusters of grapes. Other genera in the family in-

The contributions of J. Kirk Skeeles to previous editions are acknowledged.

Table 23.1. Staphylococcal-related infections in poultry.

Location	Age	Lesion	Usual outcome
Bone	Any, usually older	Osteomyelitis	Lameness
Joint	Any, usually older	Arthritis/Synovitis	Lameness
Yolk sac	Chicks, poults	Omphalitis	Death
Blood	Any	Generalized necrosis	Death
Skin	Young	Gangrenous dermatitis	Death
Feet	Mature	Bumblefoot	Lameness

clude *Gemella, Macrococcus, and Salinicoccus* (40). *Macrococcus* and *Salinicoccus* are considered to be nonpathogenic. *Gemella* spp. have, in rare cases, been involved in human disease (76, 118).

A number of *Staphylococcus* species have been isolated from the skin and nares of healthy poultry, including *S. aureus, S. epidermidis, S. xylosus, S. cohnii, S. lentus, S. saprophyticus, S. sciuri,* and *S. gallinarum* (31–33, 63, 90, 102, 109). *S. aureus* is the most common species isolated from poultry that are ill (13, 14, 17, 19, 56, 77, 87, 121, 131). Other species are also found occasionally and may in some cases be opportunists. In one outbreak of systemic illness, the major staphylococcal species isolated from the liver, blood, and hock of sick 6-week-old chickens included *S. lentus, S. simulans, S. cohnii, S. gallinarum,* and *S. capitis* (8). In this study, *S. aureus* was uncommon. *S. hyicus* has been associated with fibrinoheterophilic blepharitis in chickens and turkeys (19) and was isolated from 5 of 9 tibiotarsal growth plates of turkeys with stifle joint osteoarthritis (112). *S. sciuri, S. simulans, S. epidermidis, S. lentus, S. warneri, S. cohnii,* and *S. intermedius* have been reported from scabby hip lesions in broiler chickens (102). Other staphylococci found in human beings and domestic animals are not known to be important pathogens in poultry.

Morphology and Staining

Typical staphylococci are gram-positive, coccoid in shape, and found in clusters when grown on solid media. In liquid media, they may occur in short chains. Older cultures (> 24 hours) may stain gram-negative.

Growth Requirements

Staphylococci are readily isolated on 5% blood agar with growth evident in 18–24 hours.

Colony Morphology

S. aureus is considered to be the most pathogenic staphylococcal species in poultry and is isolated from the majority of clinical infections. Within 24 hours, aerobic growth of *S. aureus* results in circular, smooth, ß-hemolytic colonies, 1–3 mm in diameter, which are often pigmented white to orange (130). Colonies of coagulase-negative staphylococci are similar but are often gray to cream or white and nonhemolytic.

Biochemical Properties

S. aureus is aerobic, facultatively anaerobic, ß-hemolytic, usually coagulase-positive, catalase-positive, fermentative for glucose and mannitol, and gelatinase-positive. *S. hyicus* is similar biochemically to *S. aureus* but some strains have a delayed positive coagulase reaction. Most other staphylococci found in poultry are coagulase negative. Coagulase-negative staphylococci can be identified to the species level using panels of biochemical tests (23, 34, 68), automated systems (23, 66), or genetic testing (23, 26, 36, 44, 81, 110). However, this is infrequently done in clinical laboratories.

Susceptibility to Chemical and Physical Agents

Staphylococci are extremely hardy and remain viable for long periods of time on solid media or in exudates. Some strains are heat and disinfectant resistant (80). A resistance feature used to isolate *S. aureus* from heavily contaminated clinical material is its tolerance to high (7.5%) concentrations of NaCl (67, 97).

Antigenic Structure and Toxins

The antigenic nature of *S. aureus* is often complex. Strains may have a capsule consisting of glucosaminouronic acid, manosaminouronic acid, lysine, glutamic acid, glycine, alanine, or glucosamine; polysaccharide A consisting of linear ribitol teichoic acid, N-acetylglucosamine, and D-alanine; and protein-A, a cell-wall component that interacts nonspecifically with the Fc portion of immunoglobulin and may be a virulence factor. A variety of enzymes and toxins including hyaluronidase (spreading factor), deoxyribonuclease, fibrinolysin, lipase, protease, hemolysins, leukocidin, dermonecrotic toxin, hemolysins, exfoliative toxins, and enterotoxins also can contribute to a strain's pathogenicity and virulence (2, 6, 85, 130). Toxic shock syndrome toxin 1 (TSST-1) has also been found in *S. aureus* isolated from chickens, but there is currently no evidence that this toxin is directly linked to disease in poultry (65).

Strain Classification

Phenotyping techniques, such as biotyping and phage typing, have been used to classify poultry *S. aureus*. Biotyping can determine the origin and epidemiological connections of *S. aureus* isolates as host-specific (human being or domestic animal) ecovars (29, 52) or nonhost-specific biotypes (107). Phage typing has been used for poultry and human *S. aureus* strains (46, 104, 105, 106, 107). In poultry, phage typing has been used to determine the association between country of origin (Europe, Australia, Argentina, Japan) and pathogenic and nonpathogenic strains (46, 64, 104, 107, 116), but 2.2–25.8% of chicken *S. au-*

reus remain nontypable (107). Phages tend to be specific for *S. aureus* of poultry origin and cannot be used to type strains from other species (106). Genomic fingerprinting by pulsed-field gel electrophoresis is also a useful method for discriminating poultry *S. aureus* strains and for subtyping strains of avian phage groups or poultry-specific ecovars (16, 55, 107). This technique was able to type all chicken *S. aureus* strains, including those that were not phage typable (107).

Strains also have been classified using antibiotic susceptibility patterns, plasmid profiles (67), and serotyping based on capsular polysaccharides (25). Chicken capsular types were type 5 (91%) and type 8 (9%), and turkey capsule types were type 5 (33%), type 8 (38%), and nontypable (29%) (25).

Virulence Factors

Coagulase-positive isolates of *S. aureus* are considered to be pathogenic for poultry. Coagulase-negative strains are often nonpathogenic in poultry but can be pathogenic in some species.

Pathobiology and Epidemiology

Incidence and Distribution

Staphylococcus spp. are ubiquitous, normal inhabitants of skin and mucous membranes and are common environmental organisms where poultry are hatched, reared, or processed. Most staphylococcal species are considered to be normal flora, which suppress other potential pathogens through interference or competitive exclusion. Some have the potential to be pathogenic and produce disease, if allowed entry through the skin or mucous membranes.

Staphylococcus spp. and staphylococcosis have been associated with poultry throughout the world including Argentina (114), Australia (64), Belgium (27, 28), Bulgaria (10), Canada (88), China (18), Costa Rica (86), France (129), Germany (70, 71), Hungary (47), India (98), Italy (50), Japan (107), the Netherlands (96), Pakistan (117), Poland (133), Romania (84), Taiwan (122), the United Kingdom (116), and the United States (58).

Natural and Experimental Hosts

All avian species are susceptible to staphylococcal infections.

Transmission, Carriers, and Vectors

The pathogenesis of *S. aureus* infections is not completely defined, but for infection to occur, a breakdown in the natural defense mechanisms of the host must occur (2, 6). In most cases, this would involve damage to an environmental barrier, such as a skin wound or inflamed mucous membrane, and hematogenous dissemination where a locus of infection (e.g., osteomyelitis) is established, usually in the metaphyseal joint (10, 13, 24, 91). The open navel of newly hatched chicks leading to omphalitis, minor surgical procedures (e.g., trimming of toes, beak, or comb; removal of the snood), and parenteral vaccinations may offer additional means of entry for staphylococci.

Another type of host defense impairment occurs following infectious bursal disease (101), chicken infectious anemia, or possibly Marek's disease, in which the bursa of Fabricius or thymus is damaged and the immune system is compromised. Under these conditions, septicemic staphylococcal infections can occur and cause acute death. Gangrenous dermatitis caused by *S. aureus*, either with or without *Clostridium septicum* (127), can be seen following early infectious bursal disease virus infection (43, 100).

Escherichia coli was discovered to be the predominant bacterial organism in the livers of turkeys immediately following challenge with virulent hemorrhagic enteritis virus (HEV). However, when livers of survivors were cultured 2 weeks post-exposure, *Staphylococcus* spp. were the predominant bacteria (92). This suggests HEV, and possibly other similar viral intestinal infections, may create portals of entry and provide the underlying basis for subsequent staphylococcal problems associated with older, commercial turkeys.

Susceptibility to staphylococcal infections also may be genetically influenced. Two related lines of New Hampshire chickens had significant differences in mortality following experimental infection (22). The avian major histocompatibility complex influences the susceptibility to staphylococcal skeletal disease in chickens (59).

Incubation Period

The incubation period is short. Experimentally, chickens can be readily infected by the intravenous route but not as well by the intratracheal or aerosol routes (58). In experimental infections of chickens, clinical signs were evident 48–72 hours following intravenous inoculation, but the severity of lesions was dose dependent (4). The ability to consistently produce experimental disease is dependent on the number of intravenously administered bacteria (4). At least 10^5 organisms/kg body weight are necessary (88, 89).

Clinical Signs

Early clinical signs include ruffled feathers, lameness in one or both legs, drooping of one or both wings, reluctance to walk, and fever (88). This can be followed by severe depression and death. Birds surviving the acute disease have swollen joints, sit on their hocks and keel bone, and are reluctant or unable to stand (39, 88). Clinical signs of septicemic staphylococcal infection and gangrenous dermatitis occur in birds in good condition and may be evident only because of increased mortality in the flock (14, 43, 100).

Morbidity and Mortality

Morbidity and mortality due to staphylococcosis is usually low, even in the face of septicemia, unless there has been massive contamination of chicks because of exposure to unusually high numbers of bacteria in the hatchery environment or through vaccination or service procedures. Reluctance to walk to feeders and waters can lead to debilitation and death. Several reports from diagnostic laboratories have indicated that *S. aureus* is the most common bacterial agent isolated from infected legs and joints (62, 69). The number of chickens that develop gangrenous dermatitis is low, but usually all chickens that develop lesions succumb to the infection (15, 43, 69).

Pathology

Gross

Gross lesions of osteomyelitis in bone consist of focal yellow areas of caseous exudate or lytic areas (Fig. 23.1A), which cause affected bones to be fragile. Bones and sites most frequently involved are the proximal tibiotarsus and proximal femur. Less commonly, the proximal tarsometatarsus, distal femur, distal tibiotarsus, proximal humerus, ribs, or vertebrae may be involved. In affected birds, the femoral head often separates from the shaft by a fracture through the neck when the coxofemoral joint is disarticulated (femoral head necrosis) (Fig. 23.1C) (88, 91).

Arthritis, periarthritis, and synovitis are common. Affected joints are swollen and filled with inflammatory exudate as the infection (osteomyelitis) extends from nearby metaphyseal areas (Fig. 23.1D) (83, 91). Spondylitis involving articulating thoracolumbar vertebrae may cause lameness indirectly because of the impingement on the spinal cord (17, 91).

Gross lesions of septicemic staphylococcal infection consist of necrosis and vascular congestion in many internal organs including the liver (Fig. 23.1E), spleen, kidneys, and lungs (14). Dark, moist areas under the skin with crepitation are seen in gangrenous dermatitis (14, 43). Following mild trauma, gangrenous dermatitis lesions develop on the wing tips of birds infected with chicken infectious anemia virus. This condition has been referred to as "blue-wing disease."

Staphylococcal-related hatchery infections are common and can cause increased mortality within the first few days after hatching. Chicks have wet navel areas and deteriorate rapidly. Internally, the yolk sacs are enlarged with abnormal color and consistency.

Plantar abscess ("bumblefoot") is a common infection seen in mature chickens that leads to massive swelling of the foot and lameness.

Partially, or less commonly, entirely green-discolored livers (Fig. 23.1F) have been associated with osteomyelitis and/or associated soft tissue lesions (e.g., arthritis, periarthritis, tenosynovitis) in commercial turkeys at processing; this condition is called green-liver osteomyelitis complex. Carcasses with lesions from which staphylococci or other bacteria are isolated also have liver discoloration, but frequently turkeys with liver discoloration do not have demonstrable osteomyelitis or associated lesions, or bacteria cannot be isolated from the lesions (12, 20).

Liver spots are another common cause of condemnation in commercial turkeys, but most affected livers yield no aerobic or facultatively anaerobic bacteria. In one study, *S. cohnii* and other staphylococci were isolated most frequently from the few culture-positive livers in 2 flocks with histories of high liver condemnation (95). Ascarid larval migration appeared to be the most likely cause of the liver lesions(95).

Microscopic

Histologically, staphylococcal lesions consist of necrosis; bacterial colonies are composed of large numbers of gram-positive, coccoid bacteria and heterophils (Fig. 23.1B) (6, 38, 48, 88). Supernatants from pathogenic *S. aureus* resulted in increased chemotaxis of heterophils compared to supernatants from nonpathogenic *S. xylosus*; this appears to correlate with the heterophilic infiltrate seen in staphylococcal lesions (5). Longstanding lesions are primarily granulomatous.

Immunity

Neither active nor passive immunity appears to be effective in preventing *S. aureus* infections in poultry. It has been implied that specific antibody to *S. aureus* may promote the development of *S. aureus*-related infections in chickens (42, 49). Additionally, anti-staphylococcal antibodies may not significantly increase the opsonization and phagocytosis of *S. aureus* compared to the naturally exposed complement-activating cell wall materials during infection (3). Whole-cell bacterins and toxoids have not proven to be effective in other species (2, 6, 85). *S. aureus* vaccines directed against cell wall components, such as peptidoglycan and teichoic acid, or capsular polysaccarides have been used in other species with variable results (3, 108).

Diagnosis

Isolation and Identification of Causative Agent

Staphylococcosis is diagnosed by culturing suspected clinical material including exudate from joints, yolk material, and stab swabs of internal organs. The basic medium for growing staphylococci is blood agar (preferably sheep or bovine). Organisms grow well with colonies 1–3 mm in diameter within 18–24 hours. Most *S. aureus* strains are ß-hemolytic; other staphylococci are usually nonhemolytic. Heavily contaminated material should be streaked onto a selective medium inhibitory for gram-negative bacteria, such as mannitol-salt or phenylethyl-alcohol agar (67, 97, 130).

Most *S. aureus* colonies will be pigmented, while most other staphylococcal colonies are gray to white. Colonies should be selected and gram stained. Staphylococci are gram-positive cocci. Biochemical tests such as the catalase test can differentiate staphylococci from other gram-positive organisms such as *Streptococcus*. Coagulase and mannitol fermentation tests are useful in the presumptive identification of *S. aureus*. The coagulase test is commonly used to differentiate *S. aureus* from coagulase-negative staphylococci such as *S. epidermidis* (Table 23.2). A few other staphylococcal species including *S. intermedius, S. hyicus, S. lugdunensis, S. schleiferi*, and *S. delphini* may also be coagulase positive (11) but these species are uncommonly associated with clinical disease in chickens. Unlike most other staphylococci, *S. aureus* also ferments mannitol. Panels of

Table 23.2. Differentiation of *Staphylococcus aureus* and *S. epidermidis.*

Characteristic	*S. aureus*	*S. epidermidis*
Colony pigment	+	–
Hemolysis	+	–
Coagulase	+/–	
D. mannitol fermentation	+	–

biochemical tests (23, 34, 68) and genetic testing (23, 26, 36, 44, 81, 110) can be used for the definitive identification of staphylococci to the species level; however, this is rarely done in clinical laboratories. Commercially available systems can also be used (23, 66), but these systems may have difficulty identifying some species from veterinary specimens (124).

Serology

Serology is not generally used for the diagnosis of staphylococcosis, but a microtiter plate agglutination assay (3, 42) and an indirect immunofluorescent antibody titer assay have been described (3). Both have primarily been used in research.

Differential Diagnosis

Staphylococcosis can resemble infection with *E. coli, Pasteurella multocida, Salmonella gallinarum, Mycoplasma synoviae*, reoviruses, or any other infection of bones or joints that is hatchery-related, associated with mechanical trauma, or causes septicemia.

Intervention Strategies

Management Procedures

Any management procedure reducing damage to host defense mechanisms will help prevent staphylococcosis. Because wounds are a portal of entry for *S. aureus* into the body, interventions that decrease the risk of injury will also help prevent infection. Sharp objects such as splinters, jagged rocks, or metal edges that can cut or puncture the feet should be eliminated from areas where poultry are reared. Maintenance of good litter quality will reduce foot pad ulceration. Particular attention should be given to hatchery management and sanitation. *S. aureus* is ubiquitous, and conditions in incubators and hatchers are ideal for bacterial growth. Recently hatched and hatching chicks with open navels and immature immune systems can be infected easily, leading to mortality and chronic infections shortly after hatching. Prevention of early infections with infectious bursal disease virus and chicken infectious anemia virus also will help prevent staphylococcosis (101).

Poultry under mild stress are more resistant to experimental staphylococcosis than those not stressed (21, 54, 72, 88). Resistance is attributed to an increase in heterophil numbers, which occurs in birds under stress. The heterophil is thought to be the most important cell in controlling bacterial infections, particularly *S. aureus* (4, 89).

Vaccination

Staphylococcal bacterins have been ineffective in preventing infections in poultry (2, 6), but the use of live, avirulent vaccines based on the principle of bacterial interference have shown some promise. Avirulent *S. aureus* strain 502A has been used in humans to manage recurrent furunculosis and to abort nursery outbreaks (85). One strain of staphylococcus was shown to interfere with the colonization of chickens by other strains of *S. aureus* (30). Using the principle of bacterial interference, a live, avirulent vaccine for the prevention of staphylococcosis in turkeys has been developed. A naturally occurring, coagulase-negative, avirulent *S. epidermidis* isolate, designated strain 115, that colonizes cells and tissues in the respiratory tract and prevents adherence of virulent strains of *S. aureus* is used (82). In addition to interfering with the colonization of virulent *S. aureus, S. epidermidis* 115 secretes a stable, antibiotic-like bacteriocin capable of inhibiting and killing virulent *S. aureus*. The vaccine is administered by aerosol at 1–10 days and again at 4–6 weeks of age. Use of strain 115 in commercial flocks has reduced the number of turkeys with staphylococcosis and improved overall health and survival. Similar results were found when strain 115 was used in chickens (58, 74, 82, 93, 94, 128).

Competitive gut exclusion using *Lactobacillus acidophilus* was attempted to exclude *S. aureus* from experimentally infected, germ-free chickens. The treatment was effective in reducing *S. aureus* counts in crop contents, but counts in the ceca and rectum were unaffected (123).

Treatment

S. aureus infection sometimes can be treated successfully, but sensitivity tests should always be performed, because antibiotic resistance is common (28, 31, 111, 132). Drugs used successfully for treatment include penicillin, streptomycin, tetracyclines, erythromycin, novobiocin, sulfonamides, lincomycin, and spectinomycin.

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Streptococcus and Enterococcus

Stephan G. Thayer, W. Douglas Waltman, and Dennis P. Wages

Streptococcus

Introduction

Streptococcosis in avian species is worldwide in distribution, occurring as both acute septicemic and chronic infections with mortality ranging from 0.5%–50%. Infection is considered secondary, because streptococci may form part of the normal intestinal and mucosal flora of most avian species, including wild birds (5). Streptococci are ubiquitous in nature and commonly found in various poultry environments.

The previously published chapter on streptococcosis included streptococci in both Lancefield antigenic serogroups C and D. Lancefield group D *Streptococcus* spp. are commonly referred to as "fecal strep." The application of new bacteriologic techniques, especially DNA-DNA and DNA-rRNA hybridization has led to the reclassification of the Lancefield group D streptococci to the *Enterococcus* spp. (29, 64). In reviewing this chapter, the reader must keep in mind the change in reference nomenclature of both *Streptococcus* spp. and *Enterococcus* spp. Earlier studies and reports that identified bacteria by genus only could have been classified as *Enterococcus* spp. in present day classification instead of *Streptococcus* spp. When researching diseases caused by the Lancefield antigenic serogroup D, review "Enterococcosis" which follows.

History

Acute streptococcal infections of poultry were first described in chickens in 1902 (56) and 1908 (50) as apoplectiform septicemia. Chronic streptococcosis caused 50% mortality in a flock over a 4-month period (37) and was identified as the cause of mortality due to salpingitis and peritonitis in chickens (25). Streptococcosis in turkeys was reported as early as 1932 (78). Bacterial or vegetative endocarditis associated with streptococci was first reported in 1927 (63) and again in 1947 (60). A more extensive historical review of streptococcosis can be found in Peckham (58).

Etiology

The genus *Streptococcus* is composed of gram-positive, spherical bacteria occurring singly, in pairs, or short chains, which are nonmotile, non-spore-forming, facultative anaerobes. They are catalase-negative and ferment sugars, usually to lactic acid. The relationship of these characteristics to pathogenicity is unknown. *Streptococcus* spp., isolated from avian species and associated with disease, includes *S. zooepidemicus* (occasionally referred to as *S. gallinarum*) from Lancefield antigenic serogroup type C, *S. bovis*, and *S. dysgalactiae*. A new species, *S. pleomorphus,* an obligate anaerobe in normal cecal contents of chickens, turkeys, and ducks, has also been described. Its possible role in disease for these species is undetermined (1). *S. mutans*, a common bacterium in the human oral cavity, has been associated with septicemia and mortality in geese. Contaminated drinking water and poor quality litter were possible predisposing factors (40).

Both experimental and naturally occurring infections of *S. bovis* causing acute septicemia and joint infections have been found in racing pigeons (20, 22).

S. dysgalactiae has been cultured from broilers with cellulitis, a condition observed on the skin and subcutaneous tissue at processing (77).

Streptococcus spp. has been isolated from lesions of osteomyelitis in turkeys, along with *E. coli* and *Staphylococcus* spp. (17).

Naturally occurring and experimental poultry infections resulting in bacterial endocarditis commonly are associated with streptococci and other bacteria. These include *S. zooepidemicus* (51), *S. gallinaceous* (11), *E. faecalis* (13, 25, 41), *E. faecium* (25, 65), *E. durans* (13, 25), *Staphylococcus aureus*, and *Pasteurella multocida* (33).

Thanks to Chris Herron of the Educational Resources Center, College of Veterinary Medicine, The University of Georgia, Athens, Georgia, for the preparation of the color photographs.

Pathobiology and Epidemiology

S. zooepidemicus occurs almost exclusively in mature chickens but has been documented as a cause of mortality in wild birds (41). Experimentally, rabbits, mice, turkeys, pigeons, ducks, and geese are susceptible.

Transmission of streptococci occurs most commonly via oral and aerosol routes. Transmission can occur, however, through skin injuries, especially in caged layers. Aerosol transmission of *S. zooepidemicus* results in acute septicemia in chickens. Incubation periods range from 1 day to several weeks, with 5–21 days being most common.

Endocarditis occurs when septicemic streptococcal infection progresses to a subacute or chronic stage (42).

Clinical Signs

With *S. zooepidemicus* infections, clinical signs are typical of an acute septicemic infection and include lassitude, bloodstained tissue and feathers around the head, yellow droppings, emaciation, and pale combs and wattles. Cyanosis in the terminal stages has also been described (58). Mortality can range from low to 50%. Chickens in production may have an egg production drop of as much as 15%.

Streptococcus spp. and *Staphylococcus aureus* have been isolated in cases of acute fibronopurulent conjunctivitis (10).

In pigeons, *S. bovis* infection produced the acute onset of mortality with occasional lameness, inappetence, diarrhea, and the inability to fly (20).

Pathology

Gross

Gross lesions of *S. zooepidemicus* in acute disease are characterized by splenomegaly, hepatomegaly (with or without miliary to 1 cm red, tan, or white foci), enlarged kidneys, congestion of subcutaneous tissue, and peritonitis. Subcutaneous and pericardial fluid may appear serosanguineous. Bloodstained feathers around the mouth and head with blood coming from the mouth have been described (50, 58). In pigeons, congestion of the spleen and liver with accumulations of fluid around the pectoral muscles has been observed (20). In broilers, cellulitis involving the skin and subcutaneous tissues observed at processing has been associated with both *Escherichia coli* and *S. dysgalactiae* (77).

Microscopic

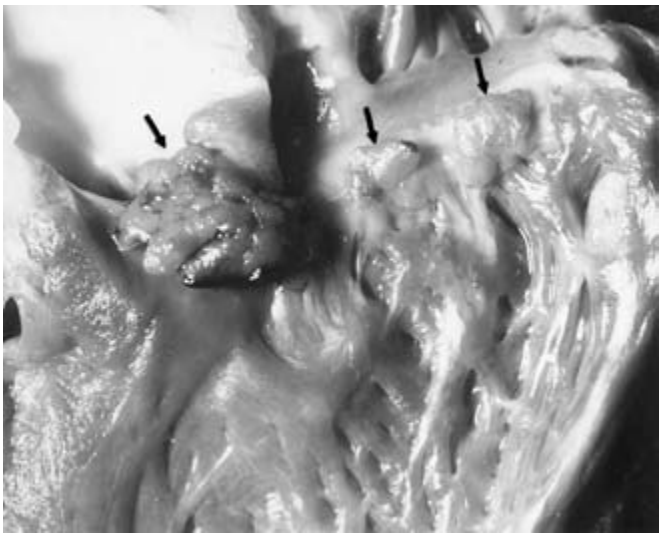
Microscopically, the liver has dilated sinusoids congested with red blood cells and increased heterophils. If foci are present grossly, there are multiple areas of necrosis and/or infarction with heterophil accumulation and thrombosis.

In experimental inoculations of pigeons with *S. bovis*, leptomeningitis and encephalitis with diffuse heterophil infiltration and perivascular cuffing are common (20).

Lesions of chronic streptococcal infections include fibrinous arthritis and/or tenosynovitis, osteomyelitis, salpingitis, fibrinous pericarditis and perihepatitis, necrotic myocarditis, and valvular endocarditis (Fig. 23.2). Vegetative valvular lesions are usually yellow, white, or tan; are small; and have raised rough areas on the valvular surface (Fig. 23.3). Valve lesions most consistently



23.2. *Streptococcus zooepidemicus* infection showing perihepatitis and peritonitis. (M. C. Peckham)



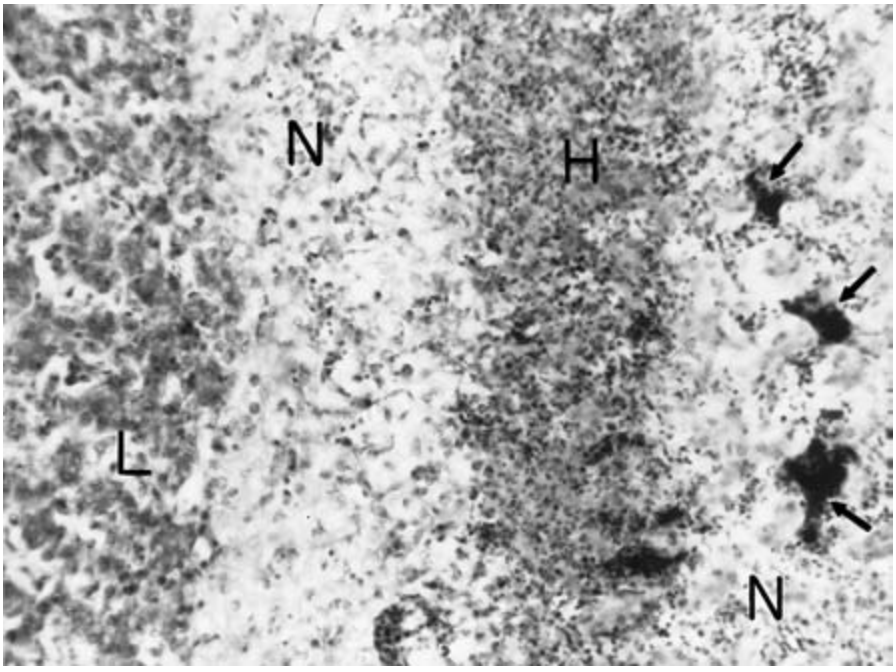
23.3. Bacterial endocarditis showing vegetations of mitral valve (arrows).



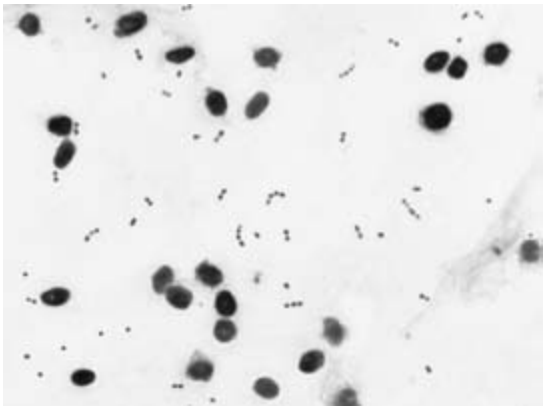
23.4. Bacterial endocarditis, showing infarcts of liver and myocardium.

are found on the mitral valve, and less frequently are found on the aortic or right atrioventricular valve. Additional gross lesions associated with valvular endocarditis include enlarged, pale, flaccid heart; pale to hemorrhagic areas in the myocardium, especially at the base of the valves, below the affected valve, or apex of the heart; infarcts in the liver, spleen, or heart and, less commonly, infarcts in the lung, kidney, and brain. Infarcts can be light colored or hemorrhagic with sharp margins. In the liver, infarcts usually are located near the ventral and posterior margins and are well demarcated, extending beneath the capsule into the parenchyma (31) (Fig. 23.4). Lesions of longer duration tend to have a sharp, narrow, lighter colored band just inside the infarct margin (42).

Microscopically, valvular lesions consist primarily of fibrin with bacteria, heterophils, macrophages, and fibroblasts. There is interstitial edema and infiltrative valvular distortion, with focal deposition of platelets and fibrin and subsequent microbial growth (35, 42). Cardiac histiocytes (Anichkov's myocytes) are numerous in the fibrous portion of the valve. Events leading to the vegetative valve lesion are 1) edema that loosens the valve surface epithelium, 2) fibrin deposition, and 3) bacterial attachment to the fibrin and colony formation. Other microscopic lesions related to endocarditis include cerebral vasculitis and infarcts, leptomeningitis, glomerulonephritis, and thrombosed pulmonary vessels (42). Cerebral lesions usually are confined to the corpus striatum. Focal granulomas can be found in virtually any tissue as a result of septic emboli. Liver infarcts are characterized by portal venous thrombosis followed by necrosis. Aggregates of bacteria are present throughout necrotic areas with a zone of heterophils just within the necrotic border, a characteristic feature of the lesion (Fig. 23.5). Gram-positive bacterial colonies are observed readily in thrombosed vessels and within necrotic foci with tissue gram stains.



23.5. Margin of liver infarct associated with bacterial endocarditis, showing clumps of bacteria (arrows), necrotic area (N), zone of necrotic heterophils (H), and relatively normal liver tissue (L). H & E, ×400.



23.6. *Streptococcus zooepidemicus* in blood of naturally infected chicken. Gram, ×800. (23)

Diagnosis

Isolation and Identification of Causative Agent

Demonstration of bacteria typical of streptococci in blood films (Fig. 23.6) or impression smears of affected heart valves or lesions from birds with typical signs and lesions will provide a presumptive diagnosis of streptococcosis.

Isolation of *S. zooepidemicus*, or any other Lancefield serogroup C streptococci from typical lesions in poultry with appropriate clinical signs, will confirm streptococcosis. Streptococci are isolated easily on blood agar. The lack of growth on MacConkey agar, different types of hemolysis, variable PYR (pyrrolidonylarylamidase) reaction and variable reactivity on bile esculin agar help to differentiate the avian streptococci. The fermentation of mannitol, sorbitol, arabinose, sucrose and raffinose are useful in the speciation of Lancefield serogroup D streptococci. *S. zooepidemicus* and other Lancefield serogroup C streptococci can be further differentiated using additional conventional tests or automated systems(38, 51, 81). Preferred tissues for culture include liver, spleen, blood, yolk, embryo fluids, or any suspected lesion area. Diagnosis of bacterial endocarditis can be made based on valvular vegetations with secondary infarcts of myocardium, liver, and/or spleen. In suspected cases, it is important to culture lesions to establish a definitive diagnosis and rule out other bacteria.

Serology

A rapid detection test by latex agglutination has been described for the identification of antigenic serogroup C streptococci in animals (38).

Differential Diagnosis

Differential diagnosis includes other bacterial septicemic diseases (e.g., staphylococcosis, colibacillosis, pasteurellosis, and erysipelas).

Treatment

Treatment includes the use of antibiotics such as penicillin, erythromycin, novobiocin, oxytetracycline, chlortetracycline, and tetracycline in acute and subacute infections. Clinically affected birds respond well early in the course of the disease. As the disease progresses within a flock, treatment efficacy decreases. Antibacterial susceptibility should be performed on bacterial isolates in any clinical cases of streptococcosis. There is no treatment for poultry with bacterial endocarditis.

In vitro susceptibility of *S. bovis* from pigeons has been demonstrated to the penicillins, macrolides, lincomycin, tetracyclines, chloramphenicol, and nitrofurans (21).

Prevention and control require reducing stress and preventing immunodepressive diseases and conditions. Proper cleaning and disinfection can reduce environmental streptococcal resident flora to minimize external exposure. The use of formaldehyde reduces the total count of *Streptococcus* spp. in the hatchers by as much as 85.7%. Reduction of 7 log-10 bacterial counts has also been demonstrated compared with ozone use, which had bacterial-count reduction of 4 log-10 (82).

Enterococcus

Introduction

The previous chapter on streptococcosis included streptococci in both Lancefield antigenic serogroups C and D. Lancefield group D *Streptococcus* spp. are commonly referred to as "fecal strep" (35). The application of new bacteriologic techniques, especially DNA-DNA and DNA-rRNA hybridization, has led to the reclassification of the Lancefield group D streptococci to the *Enterococcus* spp. (29, 64). This chapter reviews the diseases specific to *Enterococcus* spp. In reviewing this chapter, the reader must keep in mind the change in reference nomenclature of both *Streptococcus* spp. and *Enterococcus* spp. Earlier studies and reports that identified bacteria by genus only could have been classified as *Enterococcus* spp. in present-day classification instead of *Streptococcus* spp., identified in previous chapter nomenclature. When researching diseases caused by the Lancefield antigenic serogroup C and other *Streptococcus* spp., the streptococcosis chapter in this book should be reviewed.

Enterococcus spp. in avian species is worldwide in distribution. Enterococci are ubiquitous in nature and commonly found in various poultry environments. *Enterococcus* spp. are considered normal microflora of the intestinal tract of poultry (23). Though found in the intestinal tract of chickens comparatively few enterococci can be found in the litter (47). A low percentage (16.67%) of poultry meat contamination with *E. faecalis* has been found in ready-to-cook products; however, no incrimination of food poisoning in humans has been found (34). However, later studies found a much higher percentage of gram-positive cocci including *Enterococcus* spp. present on meat samples at processing (67).

History

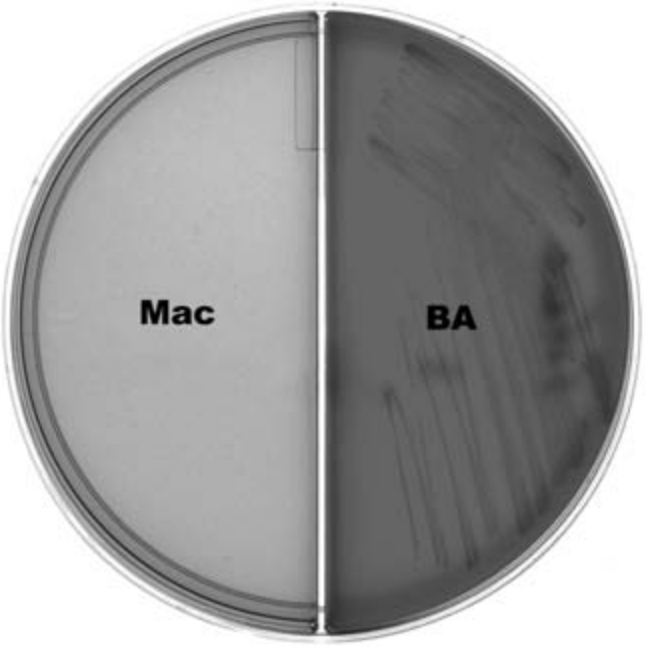
The history of *Enterococcus* spp. infections is minimal due to the inclusion of the Lancefield antigenic serogroup D in the *Streptococcus* genus. Early reports of "fecal strep" infections in poultry were reported as early as 1947 (58), 1956 (3), 1962 (33), and 1971 (42). Most of these reports named the enterococci in

the *Streptococcus* species, which makes the confirmation of earlier reports difficult. Most of the earlier reported cases involved bacterial endocarditis, hepatic granulomas, and occasionally acute septicemia.

Etiology

The genus *Enterococcus* is composed of gram-positive, spherical bacteria occurring singly, in pairs, or short chains, which are nonmotile, nonspore-forming, facultative anaerobes. They are catalase-negative and ferment sugars usually to lactic acid with a significant acid pH shift. Common avian isolates can be separated by their differential ability to ferment mannitol, sorbitol, L-arabinose, sucrose, and raffinose and by their lack of growth on MacConkey agar containing crystal violet (Fig. 23.7) (Table 23.3)(81). The relationship of these characteristics to pathogenicity is unknown. *Enterococcus* spp. isolated from avian species and associated with disease includes *E. faecalis, E. faecium, E. durans, E. avium*, and *E. hirae* from Lancefield antigenic serogroup D streptococci. In this chapter, *E. faecalis* subsp. *faecalis, E. faecalis* subsp. *liquefaciens*, and *E. faecalis* subsp. *zymogenes* will all be considered as *E. faecalis*. Other enterococci species have also been isolated from poultry (14, 18).

Bacterial endocarditis, commonly associated with both streptococci and enterococci, can be caused by many different bacteria in naturally occurring and experimental poultry infections. These include *E. faecalis* (23, 30, 39), *E. faecium* (67), *E. durans* (26), *E. hirae* (14), *Streptococcus zooepidemicus, Staphylococcus aureus*, and *Pasteurella multocida* (33). Of the enterococci isolated from naturally occurring infections, *E. faecalis* has been the most common isolate and the one most consistent in producing bacterial endocarditis in experimental infections via the intravenous route.



23.7. *Enterococcus faecalis* on a Blood agar/MacConkey agar bi-plate demonstrating growth on blood agar and no growth on MacConkey agar containing crystal violet.

Pathobiology and Epidemiology

E. faecalis affects species of all ages; it is a serious disease occurring in embryos and young chicks from fecally contaminated eggs (3, 4). *E. faecium* has been identified as a cause of mortality in ducklings (67).

Transmission of enterococci occurs most commonly via oral and aerosol routes. Transmission can occur, however, through skin injuries, especially in caged layers. Most enterococci are pathogenic when administered intravenously. Aerosol transmission of *E. faecalis* results in acute septicemia in chickens (3). High mortality from acute septicemia and liver granulomas occur after experimental oral inoculation with *E. faecalis* (35). *E. faecalis* has been incriminated as the cause of loss of intestinal epithelium integrity allowing bacteria (e.g., *Bacteroides* spp., *Catenabacterium* spp., *Eubacterium* spp., and *Streptococcus* spp.) to produce liver granulomas in turkeys (55). These bacteria and *Proprionibacterium* spp., *Corynebacterium* spp., *Staphylococcus* spp., and *Lactobacillus* spp. can often be isolated from turkey liver granulomas. Concurrent enteric infections, or any condition compromising the intestinal villous epithelium and allowing penetration of resident enterococci, can result in septicemia and/or bacterial endocarditis. Incubation periods range from 1 day to several weeks, with 5–21 days being most common.

Table 23.3. *Enterococcus* differential fermentation characteristics.

	Fermintation of				
Species	**Mannitol**	**Sorbital**	**L-arabinose**	**Sucrose**	**Raffinose**
E. avium	+	+	+		
E. durans	–	–	–	–	–
E. faecalis	+	+	–		
E. faecium	+	–	+		
E. hirae	–	–	–	+	+

*** Enterococcus does not grow on MacConkey agar containing crystal violet.*

Experimental bacterial endocarditis (vegetative or valvular) results from intravenous exposure. *E. faecalis* isolates and other species of enterococci from intestines of apparently normal birds can produce endocarditis (33, 53, 60, 62). Endocarditis can occur when a septicemic enterococcal infection progresses to a subacute or chronic stage (42).

Chicks experimentally inoculated with *E. faecalis* had higher retained yolk weights, higher total plasma protein levels, and decreased Newcastle disease antibody passive transfer (absorption) through infected yolk sacs (65).

Enterococcus spp. has been associated with brain necrosis and encephalomalacia in young chickens (2, 11, 15, 24).

Some enterococci, however, have been demonstrated to have a beneficial effect on growth and feed efficiency (57) and are the subject of research as potential probiotics.

E. faecalis has been reported to be a bacterial component of amyloid arthropathy in chickens (48, 49).

Clinical Signs

Enterococcus spp. in poultry can result in 2 distinct clinical forms of disease, acute and subacute/chronic. In the acute form, clinical signs are related to septicemia and include depression, lethargy, lassitude, pale combs and wattles, ruffled feathers, diarrhea, fine head tremors, and decrease or cessation of egg production. Often, only dead birds are found.

In the subacute/chronic form, depression, loss of body weight, lameness, and head tremors may be observed. Chickens experimentally inoculated intravenously with *E. faecalis* develop leukocytosis 2–3 days postinoculation; highest values occur in birds that develop endocarditis (33). Heterophils predominate, along with a slight monocytosis. Body temperatures are elevated in birds with persistent bacteremia. Numbers of bacteria present in peripheral blood vary considerably. Clinically affected birds eventually die if not treated.

Egg transmission or fecal contamination of hatching eggs with enterococci may result in late embryo mortality and an increased number of chicks or poults unable to "pip" or penetrate through the shell at hatch (4). Overall bacterial contamination, including *Enterococci* spp., at the time of hatching can contribute to mortality of chicks early in life (65).

Pathology

Gross

Gross lesions of enterococci in acute disease are characterized by splenomegaly, hepatomegaly (with or without foci), enlarged kidneys, and congestion of subcutaneous tissue. Omphalitis and/or enlarged yolk sacs are observed in chicks or poults infected at hatching (4, 66, 40). Hepatomegaly, splenic necrosis, fibrinous pericarditis, perihepatitis, and airsacculitis were observed in ducks infected with *E. faecium* (67).

Lesions of chronic enterococcal infections include fibrinous arthritis and/or tenosynovitis, osteomyelitis, fibrinous pericarditis and perihepatitis, necrotic myocarditis, and valvular endocarditis. Vegetative valvular lesions are usually yellow, white, or tan; are small; and have raised rough areas on the valvular surface. Valve lesions most consistently are found on the mitral valve, and less frequently on the aortic or right atrioventricular valves. The changes are similar to those observed with *S. zooepidemicus* infection. Additional gross lesions associated with valvular endocarditis include enlarged, pale, flaccid heart; pale to hemorrhagic areas in the myocardium, especially at the base of the valve, below the affected valve, or at the apex of the heart (42); infarcts in the liver, spleen, or heart; and, less commonly, infarcts in the lung, kidney, and brain. Infarcts can be light colored or hemorrhagic with sharp margins. In the liver, infarcts usually are located near the ventral and posterior margins and are well demarcated, extending beneath the capsule into the parenchyma (33). Lesions of longer duration tend to have a sharp, narrow, lighter colored band just inside the infarct margin (42).

Microscopic

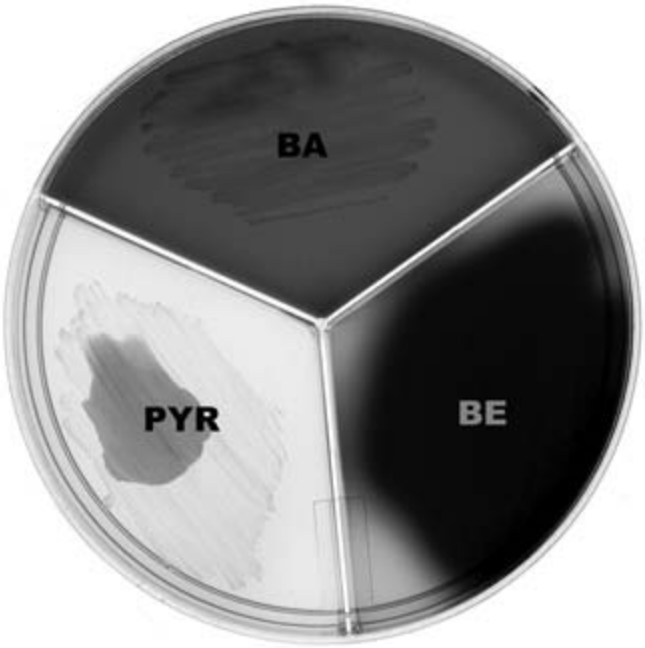
Microscopically, the liver has dilated sinusoids congested with red blood cells and increased heterophils. If foci are present grossly, there are multiple areas of necrosis and/or infarction with heterophil accumulation and thrombosis. Splenomegaly is characterized by congestion and hyperplasia of cells in the mononuclear phagocytic system (35).

Microscopically, valvular lesions consist primarily of fibrin with bacteria, heterophils, macrophages, and fibroblasts. There is interstitial edema and infiltrative valvular distortion, with focal deposition of platelets and fibrin and subsequent microbial growth (33, 42). Cardiac histiocytes (Anichkov's myocytes) are numerous in the fibrous portion of the valve. Other microscopic lesions related to endocarditis include cerebral vasculitis and infarcts, leptomeningitis, glomerulonephritis, and thrombosed pulmonary vessels (39). Cerebral lesions usually are confined to the corpus striatum. Focal granulomas can be found in virtually any tissue as a result of septic emboli. Liver infarcts are characterized by portal venous thrombosis followed by necrosis. Aggregates of bacteria are present throughout necrotic areas with a zone of heterophils just within the necrotic border; a characteristic feature of the lesion, gram-positive bacterial colonies, are readily observed in thrombosed vessels and within necrotic foci with tissue gram stains.

Diagnosis

Demonstration of bacteria typical of enterococci in blood films or impression smears of affected heart valves or lesions from birds with typical signs and lesions will provide a presumptive diagnosis of enterococcosis.

Isolation of *Enterococcus* spp. (without fecal contamination) from typical lesions in poultry with appropriate clinical signs will confirm enterococcosis. Enterococci are easily isolated using blood agar and MacConkey agar as primary isolation media (26, 81). There is a distinct lack of growth of the enterococci on MacConkey agar due to the presence of crystal violet in the more modern formulations (Fig. 23.7). For contaminated specimens the addition of PEA (phenylethanol) agar to the standard battery of media provides selectivity for gram positive organisms. Presumptive identification of enterococci can be accomplished noting gamma (no) hemolysis on blood agar, black precipitate on bile esculin agar and a positive reaction on PYR



23.8. *Enterococcus faecalis* on a tri-plate with blood agar, bile esculin agar, and PYR agar. Note gamma (none) hemolysis on blood agar, black precipitate on bile esculin agar, and magenta color which developed after addition of PYR reagent to 18–24 hour culture. This combination of reactions is indicative of Group D Enterococcus. Speciation requires carbohydrate testing. See table 23.3.

(pyrrolidonyl-beta-naphthalamide) agar (51) (Fig. 23.8). Further speciation can then be accomplished using *differential fermentation* of mannitol, sorbitol, arabinose, sucrose, raffinose (Table 23.3). Numerous commercial systems are available for bacterial identification but care should be used in interpretation or acceptance of identifications. Suspected inaccuracies should always be backed up with secondary methods often including conventional biochemical tests (39, 69, 72). Preferred tissues for culture include liver, spleen, blood, yolk, embryo fluids, or any suspected lesion area. Diagnosis of bacterial endocarditis can be made based on valvular vegetations with secondary infarcts of myocardium, liver, and/or spleen. In suspected cases, it is important to culture lesions to establish a definitive diagnosis and rule out other bacteria.

Differential diagnosis includes other bacterial septicemic diseases (e.g., staphylococcosis, colibacillosis, pasteurellosis, and erysipelas).

Intervention Strategies

Prevention and control require reducing stress and preventing immunodepressive diseases and conditions. Proper cleaning and disinfection can reduce environmental enterococcal resident flora to minimize external exposure.

Treatment

Treatment can include the use of antibiotics such as penicillin, erythromycin, novobiocin, oxytetracycline, chlortetracycline, tetracycline, or nitrofurans in acute and subacute infections. Clinically affected birds respond well early in the course of the disease. As the disease progresses within a flock, treatment efficacy decreases. Novobiocin has been found to be efficacious in ducks with *E. faecium* infection (66). Dietary bacitracin decreases the incidence of some strains of enterococci in young chickens (7). Certain enterococcus strains can develop resistance after exposure to antibiotics such as tylosin, but treatment with such antibiotics may not shift the overall number of resistant organisms (20). Chickens fed certain growth-promoting antibiotics may develop greater numbers of *E. faecium* and *E. faecalis* populations in comparison to other enterococci (44, 45). Antimicrobial susceptibility should be performed on bacterial isolates in clinical cases of enterococcosis prior to the initiation of treatment. Enterococci vary in their resistance and susceptibility to growth-promoting agents (27). Chickens fed 50 grams of chlortetracycline compared to nonmedicated controls had diversity in antimicrobial resistance patterns suggesting that other factors are involved in antibiotic resistance acquisition besides feeding low-level antibiotics (52). Environmental forces, feeding schedules, stress, and interactions between different genotypes and housing influence the response of chickens to enterococcal infections (43, 68). There is no treatment for poultry with bacterial endocarditis.

Public Health Significance

Most streptococci found in avian species are considered zoonotic and have been isolated from both animal and human infections (74). Antimicrobial susceptibilities vary widely among the species of enterococci (27, 71). The use of growth promoting antibiotic feed additives has been suggested as a direct link to the development of antimicrobial resistance in enterococcal isolates in humans (6). However bans of several growth-promoting antibiotics in the European Union have failed to reduce the rate of antibiotic resistance in human enterococcus isolates (12, 32). Other scientific studies fail to prove association of antibiotic usage and the incidence of antimicrobial resistance in human enterococcus isolates and suggest there are other contributing factors (52, 19, 59).

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Erysipelas

Joseph M. Bricker and Y. M. Saif

Introduction

Definition and Synonyms

Erysipelas in birds is generally an acute, fulminating infection of individuals within a flock. The infection and disease have been reported from many different vertebrate species, either as a contaminant (fish) or an infection. The etiologic agent of erysipelas is a small gram-positive bacterium, *Erysipelothrix rhusiopathiae,* which also causes swine erysipelas and erysipeloid in humans. The organism is distributed worldwide in nature and although the disease is sporadic in poultry, endemic areas exist.

Economic Significance

In birds, its primary economic importance is as a disease of turkeys. Outbreaks of economic significance are infrequent in other avian species. Occasional losses of individual birds within a flock have been reported, and a few economically significant outbreaks in chickens and ducklings have occurred (67). Salem *et al.* (97) reported erysipelas in meat-type chickens including both breeders and broiler-type chickens. *E. rhusiopathiae* has caused outbreaks of erysipelas in pheasants, ducks, geese, guinea fowl, chukars, grebes, and emus (19, 31, 42, 44, 51, 52, 56, 88, 128).

Erysipelas not only causes death but frequently affects the fertility of the male. Further marketing losses may result from lack of finish, condemnation, or downgrading, resulting from postmortem evidence of septicemia. Affected chicken flocks have been reported to suffer depression of egg production.

Public Health Significance

Erysipeloid in humans may be a local or septicemic, and occasionally fatal, infection. Erysipeloid is considered an occupational disease affecting particularly fish handlers, butchers, kitchen workers, veterinarians, and animal caretakers.

In most cases, the disease is preceded by an injury such as a cut. Presumptive diagnoses of the infection have been made in turkey flocks as a result of infection of a turkey handler or an insemination crew member. Mutalib *et al.* (78) described suspected erysipeloid in several plant employees processing quail from a confirmed outbreak of erysipelas. Erysipeloid was also suspected in animal caretakers following an outbreak of erysipelas in caged chicken layers (77). Although localized cutaneous infections are most often observed, Dunbar and Clarridge (33) reported several cases of *E. rhusiopathiae* infection associated with a wide range of clinical symptoms.

Silberstein (114) reported endocarditis and encephalitis in humans treated with penicillin. Several reviews of *E. rhusiopathiae* as an occupational pathogen have been compiled (17, 89).

History

Sporadic cases of infection were reported in various avian species prior to 1936. Beaudette and Hudson (6) were the first to call attention to the economic significance of the disease in North American turkeys; other outbreaks were reported shortly thereafter. The disease is of economic concern to turkey growers in some parts of the United States. The infection has been reported from time to time in one or small groups of other species of birds; with the advent of widespread artificial insemination of turkeys, prevention of erysipelas became a problem facing producers of turkey hatching eggs. With more confinement growing of turkeys, it has become a less important problem, except in endemic areas.

Following the introduction of bacterins in the early 1950s and the availability of penicillin as a treatment, various programs of preventive vaccination, and/or treatment have been followed. Despite this, cases of post-insemination erysipelas occur in turkey hens.

Etiology

Classification

Name and Synonyms

E. rhusiopathiae (formerly *E. insidiosa*) belongs to the family Lactobacillaceae (18). A second genomic species, *E. tonsillarum,* was described based on DNA homology studies with type strains of *E. rhusiopathiae* (118). Some investigators have provided evidence that there may be an additional distinct genomic species other than *E. rhusiopathiae* and *E. tonsillarum* (84, 119) or perhaps 2 additional distinct new species (123). Recently, Verbarg *et al.* (130) identified a new third genomic species with a proposed species name of *Erysipelothrix inopinata.* Only *E. rhusiopathiae* is considered the causative agent of erysipelas in avian species.

Morphology and Staining

The organism stains gram-positive but tends to decolorize, a trait particularly noticeable in older cultures. *E. rhusiopathiae* is nonmotile, does not form spores, and does not stain acid-fast. The cellular morphology of the organism is variable. Cells taken from smooth colonies or isolated from the tissues of acutely infected birds are slender, straight, or slightly curved rods measuring 0.2–0.4 by 0.8–2.5 μm and may occur singly or in short chains. Organisms from older cultures or rough colonies are filamentous rods and may form masses that resemble mycelia. These filamentous rods usually appear somewhat thickened and may appear beaded following staining. The filamentous form begins to appear after several passages on artificial media. Both short rods and short filaments may be observed from a single colony (intermediate colony type). *E. tonsillarum* is morphologically indistinguishable from *E. rhusiopathiae*.

Growth Requirements

E. rhusiopathiae is facultatively anaerobic and grows readily, although sparsely, on ordinary culture media and moderately well

in thioglycollate broth and various other broths containing serum and serum components. The addition of serum to broth media supports heavier growth with a powdery sediment forming after 24 hours. Protein hydrolysates, glucose, and certain detergents, such as Tween 80, also enhance growth. The organism grows especially well in deep stabs of semisolid medium prepared by adding 0.5% agar to tryptose phosphate broth. Feist *et al.* (37) described a medium, which supported high bacterial yields, but was devoid of serum. Using 6 different strains of *E. rhusiopathiae*, Groschup and Timoney (46) indicated higher cell densities were obtained with Feist medium than with horse serum-supplemented brain heart infusion broth. However, Sato *et al.* (100) reported higher bacterial yields with tryptic soy broth supplemented with 0.3% Tris and 0.1% Tween 80 than with Feist medium or 2 different supplemented heart infusion broths. Reduced oxygen or increased carbon dioxide (5–10%) enhances growth, but neither is necessary to support growth. Smith (115) described the appearance of growth in a meat-infusion broth culture at 24 hours as "a faint opalescence..., which on shaking was resolved for the moment into delicate rolling clouds."

E. rhusiopathiae grows in a temperature range from 4°C (slow growth) to 42°C, with an optimal range of 35–37°C. The optimal pH for growth is mildly alkaline, pH 7.4–7.8. Oleic acid and riboflavin have been reported as essential for growth. The organism does not form a pellicle.

Colony Morphology

Three different colony types have been described for *E. rhusiopathiae.* Smooth colonies are dewy, colorless to bluish gray, and of pinpoint size (0.5–0.8 mm) with smooth edges. Most strains of *E. rhusiopathiae* and organisms isolated directly from infected tissues form this colony type. Some strains, however, form rough colonies that consist of long thickened filamentous rods. Rough colonies are opaque, flat, dry, and of pinhead size (1–2 mm) with irregular or lobed edges. The dissociation from smooth to rough colony type is described as intermediate colony type in which both short rods and short filaments may be identified. Most strains produce a narrow zone of alpha hemolysis in a medium containing 5–10% horse or bovine blood after 2–3 days incubation at 37°C in an atmosphere of 5–10% carbon dioxide. A test-tube brush type of growth (lateral radiating projections) occurs 48 hours postinoculation in gelatin stab culture incubated at 21°C.

Biochemical Properties

E. rhusiopathiae ferments galactose, dextrose, fructose, maltose, lactose, and levulose without gas production. *E. tonsillarum* differs in its ability to ferment sucrose (119); however, investigators have indicated that this property may not be unique to *E. tonsillarum* (84). Lead acetate agar or triple-sugar iron (TSI) agar is usually blackened, indicating hydrogen sulfide (H_2S) production, and xylose is occasionally fermented. Strains that do not produce H_2S have been isolated (10). Litmus milk occasionally is acidified slightly without coagulation. The organism is catalase negative, does not produce indole, does not reduce nitrites, is Voges-Proskauer and methyl red negative, does not hydrolyze esculin, and does not reduce 0.1% methylene blue. White and Shuman (135) found that the fermentation pattern varied with the medium, indicator, and method of measuring acid production; they stated that the most dependable medium was Andrade's base plus serum. Of 3 methods used to measure acid production (chemical indicator, change in pH, and production of titratable acidity), they found that the chemical indicator gave the most valid reproducible results.

Susceptibility to Chemical and Physical Agents

E. rhusiopathiae is fairly resistant to various environmental and chemical factors. It is very resistant to desiccation and may survive the smoking and pickling processes used for processing meat and remain viable in frozen or chilled meat, dried blood, decaying carcasses, or fish meal. Apart from tissues, it is killed at 70°C in 5–10 minutes.

E. rhusiopathiae is destroyed in a short time by a 1:1000 concentration of bichloride of mercury, 0.5% sodium hydroxide solution, 3.5% liquid cresol, or a 5% solution of phenol. *E. rhusiopathiae* is also inactivated with 0.5% formalin. The organism is resistant to 0.001% crystal violet and 0.5% potassium tellurite and can grow in the presence of 0.1% sodium azide.

Vallee (129) suggested that the organism remained viable in soil and multiplied in alkaline soils during warm weather. Wood (138), however, reported that under experimental conditions, *E. rhusiopathiae* was inactivated at different rates in soil when parameters of temperature, pH, and organic matter content were tested. Temperature exerted the greatest effect on viability. Populations of the pathogen survived 35 days at 3°C and 2 days at 30°C. Organisms did not survive longer than 11–18 days under various conditions of organic matter content and pH.

Antigenic Structure and Toxins

Essentially all strains of *E. rhusiopathiae* possess at least one or more common antigens (40, 102, 116, 139). These antigens are heat labile and consist of protein or a complex of protein, carbohydrate, and lipid (136). The cellular fatty acid profiles of several strains of *E. rhusiopathiae* and *E. tonsillarum* were determined, which demonstrated no differences between the 2 species (121). Schubert and Fiedler (103) investigated the murein component of the cell wall and determined the murein belongs to the B1delta type. Barber (5) and Nelson and Shelton (81) reported that *E. rhusiopathiae* resembles *Listeria* sp.; however, they demonstrated marked cultural differences between them. It resembles mycobacteria in that it has a high lipid content in its cell wall (almost 30%). Similar to some gram-negative bacteria, *E. rhusiopathiae* has a rather low hexosamine content but differs from them in having a limited complement of amino acids (58). *E. rhusiopathiae* produces no known toxins.

Strain Classification

Antigenicity

At present, strain classification is based for the most part on serologic, not biologic or biochemical, activity. The serotype-based scheme utilizes a heat-stable antigen (cell wall peptidoglycan) to differentiate *E. rhusiopathiae* into serotypes. These antigens are

extracted easily from the organism using acid or by autoclaving a washed whole-cell inactivated culture for 1 hour at 121°C. Determination of serotype is accomplished with a double-diffusion gel system using specific hyperimmune rabbit sera. The preferred system for serotyping *E. rhusiopathiae* isolates is a numerical system described by Kuscera (65). Using the numerical system, strains previously designated as types A or B now become types 1 and 2, respectively. Subtypes of some serotypes have been described (e.g., serotypes 1 and 2) and are designated by a number followed by a lowercase letter. Although 26 serotypes of *E. rhusiopathiae* have been described (4, 35), the identification of *E. tonsillarum* as a distinct species has led to a division of the serotyping scheme. The majority of *E. rhusiopathiae* strains isolated from poultry fall into 3 major serotypes: types 1 (both subtypes 1a and 1b) and 2 and 5 (30, 34, 128, 143).

Immunogenicity or Protective Characteristics

Traub (126) described a "soluble immunizing substance" produced by certain *E. rhusiopathiae* strains belonging to serotype 2. This soluble immunizing substance is present on the whole bacterial cell and is released into the culture medium when the organism is grown in a complex medium containing serum. Several reports confirm Traub's initial observation that not all serotype 2 strains produce effective levels of this substance for incorporation into bacterins (61, 144).

Based on the description of a soluble immunizing substance, several different approaches were employed in attempts to characterize a protective antigen (94, 125, 136). Galan and Timoney (40) described a 64,000–66,000-MW protein gene, which was cloned and expressed as a fusion protein in a heterologous vector. Groschup *et al.* (45) demonstrated that a 64,000–66,000-MW protein was protective for mice. Sato *et al.* (98) isolated 64,000- and 43,000-MW protective proteins from culture filtrates of *E. rhusiopathiae* strains representing several different serotypes. In a later report, Sato *et al.* (100) demonstrated that the concentration of protective antigen was higher in alkaline extracts of bacteria using 0.05–1% NaOH than in culture filtrates or "ultrasonicated" extracts. Makino *et al.* (69) identified a gene encoding a surface protein with a molecular weight of 69,000 designated surface protein A (SpaA). SpaA consists of a 160-residue C-terminal repeat containing 8 repeats of 20 and 19 amino acids. Each repeat begins with the dipeptide GW, which appears to bind the protein to the cell surface (71). Shimoji *et al.* (111) described a protein designated SpaA.1 whose sequence was different from SpaA at the C-terminal region and at residues 426 and 435. Monoclonal antibodies reactive to the protective antigen have been described (61, 69, 101).

Recently, Shimoji *et al.* (106) identified two adhesive surface proteins designated RspA and RspB. Both proteins were present in surface extracts of *E. rhusiopathiae* and in culture supernatants. Recombinant RspA, expressed in *E.coli* as a fusion protein, induced partial protection in mice following challenge.

Genetic or Molecular

Methodologies including molecular and genetic techniques are challenging the current serotype-based classification scheme of *Erysipelothrix* spp., but the comparison between the two approaches is not clear-cut. Using DNA-DNA hybridization Takahashi (119) reported that strains of serotypes 3, 7, 10, 14, 20, 22, and 23 are *E. tonsillarum* and that strains of *E. rhusiopathiae* belong to serotypes 1, 2, 4, 5, 6, 8, 9, 11, 12, 15, 16, 17, 19, and 21 and type N. Strains not possessing a type-specific antigen are designated type N. Strains belonging to serotypes 13 and 18 exhibited low levels of DNA homology with *E. rhusiopathiae* and *E. tonsillarum* and possibly constitute a distinct genomic species. Takeshi *et al.* (123) have suggested that strains belonging to serotypes 13 and 18 represent 2 new and distinct species of *Erysipelothrix*. The classification of strains belonging to serotypes 24, 25, and 26 were not reported in this study. Recently, however, using randomly amplified polymorphic DNA (RAPD) analysis, Okatani *et al.* (83) determined that 2 strains belonging to serotypes 15 and 16 had the RAPD pattern of *E. tonsillarum*. Furthermore, a serotype 10 strain had the RAPD profile of *E. rhusiopathiae*. Ribotyping of the 16S rRNA genes of *Erysipelothrix* strains revealed that several strains with the same serotype could belong to either genomic species (4). Kiuchi *et al.* (62) reported that the nucleotide sequences of 16S rRNA from *E. rhusiopathiae* and *E. tonsillarum* had 99.8% homology. A rapid typing method utilizing automated ribotyping has been described (84).

Chooromoney *et al.* (22) analyzed strains of *Erysipelothrix* spp. by multilocus enzyme electrophoresis and indicated that serotyping was unreliable for epidemiologic studies, although serotype analysis was useful when one electrophoretic type contained multiple serotypes or subtypes or for strains isolated from different species and of varying virulence. Bernath *et al.* (8) evaluated the protein profiles of 12 strains of *E. rhusiopathiae* using polyacrylamide electrophoresis and autoradiography and found no significant correlation between serotype and percentages of common proteins of identical molecular weight.

Pathogenicity

There is no reported correlation between serologic grouping and the biochemical pattern of strains of *E. rhusiopathiae* isolated from birds and production of the septicemic, urticarial, or endocardial form of erysipelas or of the carrier state (11, 134).

Virulence Factors

Enzymes

The enzyme hyaluronidase initially was thought to be involved in the virulence of *E. rhusiopathiae*, but studies in mice revealed no association with hyaluronidase production and the pathogenicity of a particular strain (82, 105). Neuraminidase, however, appears to correlate better with the virulence of *E. rhusiopathiae* isolates. This enzyme is produced during logarithmic growth, and the amount of enzyme activity was reported by Müller (75) to be lower in strains of lower virulence or avirulent strains compared with highly virulent isolates. No apparent relationship exists, however, between the amount of neuraminidase activity and serotype. Using a peanut lectin hemagglutination assay, Wang *et al.* (132) demonstrated that neuraminidase production was media and pH dependent and was only produced by isolates of *E. rhusiopathiae* and not by *E. tonsillarum* strains. Abrashev and

Orozova (1) identified that large glycoproteins were better inducers of neuraminidase production in cultures of *E. rhusiopathiae.* In a review of erysipelas, Wood and Henderson (140) noted that specific antibody to this enzyme from *E. rhusiopathiae* was identified in a commercial erysipelas antiserum produced in horses. Tesh and Wood (124) reported the enzyme coagulase also was produced by strains of *E. rhusiopathiae*.

Other Factors

Lachmann and Deicher (66) inferred the presence of a capsule following their discovery of a 14,000–22,000-MW surface polysaccharide. Shimoji *et al.* (107) demonstrated a capsule using transmission electron microscopy, which showed polar thickening on some *E. rhusiopathiae* strains. Other investigators have demonstrated, at least in part, that the virulence of *E. rhusiopathiae* for mice was associated with the presence of a capsule (107, 112). Shimoji et. al. (108) discovered that murine macrophages were able to phagocytose three- to four-fold more avirulent acapsular bacteria than virulent bacteria in the presence of normal serum.

Partridge *et al.* (87) cloned and characterized a bacterial stress protein designated DnaK, which was highly expressed in *E. rhusiopathiae.* A heat shock gene homolog designated *dnaJ*, located immediately 3' to the *dnaK* gene, has also been cloned (92).

Makino *et al.* (70) reported cloning a gene from *E. rhusiopathiae*, which encoded a 16,000-MW hemolysin. A review of virulence factors of *E. rhusiopathiae* has been compiled (112).

Pathobiology and Epidemiology

Incidence and Distribution

E. rhusiopathiae is worldwide in distribution. The adaptiveness of the organism is indicated by its ability to infect a wide variety of vertebrate species. It has been isolated from the tissues of birds, mammals, reptiles, and amphibians, as well as the surface slime of fish (38, 59, 79, 80).

Outbreaks of erysipelas in poultry occur sporadically, although locations exist in the world where the disease is endemic. Although the disease in turkeys has been reported more frequently among males than females, there is no evidence of differing susceptibility between sexes. Field observations suggest that the portal of entry (skin) is breached more frequently in the male, but incidence in hens has increased due to artificial insemination and frequent handling of females.

Natural and Experimental Hosts

The organism has been isolated in nature from turkeys, chickens, ducks, geese, emus, mud hens, malleefowl, eared grebes, parrots, sparrows, canaries, finches, thrushes, blackbirds, doves, a Hawaiian crow, quail, wild mallards, white storks, herring gulls, golden eagles, pheasants, starlings, peacocks, parakeets, swine, sheep, cattle, marine and freshwater fish, various captive wild birds and mammals, chipmunks, meadow and house mice, dolphins, and crocodiles (10, 12, 13, 36, 41, 44, 55, 56, 57, 60, 67, 141, 142). From the reports, it appears that the susceptibilities of various avian populations differ. Genetic resistance may play a role in susceptibility to disease based on a report of an outbreak in turkeys with different genetic backgrounds (96). Experimental hosts are the pigeon, turkey, chicken, budgerigar, mouse, and rat.

Age of Host Commonly Affected

E. rhusiopathiae is pathogenic for turkeys at any age or sex. Hollifield *et al.* (50) reported erysipelas in 2–4-day-old turkey poults following toe-trimming at a hatchery. In addition to turkeys, other avian species are susceptible to infection with *E. rhusiopathiae,* both experimentally and under field conditions, and serious losses have been reported in chickens, ducks, and geese following naturally occurring outbreaks of the disease. Experimentally, Malik (72) demonstrated that virulent cultures of *E. rhusiopathiae* administered parenterally produced a septicemia in chickens less than 14 days old. In older chickens, however, septicemia could be produced only by intrapalpebral or subconjunctival installation of the pathogen along with injury to that tissue. Administration of hydrocortisone not only increased susceptibility to *E. rhusiopathiae* but shortened the course of infection and increased mortality, thus appearing to increase pathogenicity. Shibatani *et al.* (104) were able to induce septicemia in 3-week-old chickens using an *E. rhusiopathiae* strain previously isolated from an outbreak of erysipelas in chicken layers.

Transmission, Carriers, and Vectors

The actual portal of entry and pathogenesis of *E. rhusiopathiae* infection in birds have not been definitely established though contaminated material as the source of infection and entry through breaks in the mucous membranes or skin are the most frequent precursors. Fishmeal and fish in general have been cited as probable sources of infection for avian species (43, 76). Cousquer (29) reported an outbreak of erysipelas in racing pigeons after ingestion of composted waste. Cannibalism and fighting among birds apparently result in increased losses. Permitting the carcasses of infected dead birds to remain on the premises to be picked at or eaten by penmates also increases the spread and increases losses at unpredictable rates. Though it has been reported that the organism can survive in soil and that soil may serve as a source of the organism, current evidence indicates that soil serves only as a temporary reservoir for the organism. There is no evidence of vertical or egg transmission of *E. rhusiopathiae* (74).

Infection and disease can be produced in turkeys inoculated orally with chicken embryo yolk-propagated organisms or when allowed to feed on viscera of turkeys that died from erysipelas. Experimentally, Corstvet (25) obtained up to 50% mortality in turkeys by oral inoculation of freshly isolated virulent organisms grown in chicken embryo yolk sac. Broth culture instilled orally, intranasally, or into the conjunctival sac (without damaging the membrane) was not infectious (43). Corstvet (25) and Bricker and Saif (16) found that subcutaneous (SC) inoculation of virulent cultures resulted in local multiplication followed by septicemia, with 80–100% mortality in susceptible turkeys. Parenteral injection of most avian strains of the organism regularly kills mice (*Mus musculus*), pigeons, and turkeys, but guinea pigs and chickens usually survive. Iliadis (53) reported that pigeons were more susceptible to intravenous than to oral inocula-

tion. Takahashi *et al.* (120) reported that none of 14 *E. tonsillarum* strains tested induced any signs of disease or lesions when inoculated intramuscularly (IM) into white leghorn chickens and suggested that *E. tonsillarum* should not be considered to be a potential pathogen for chickens.

It is of interest to note that difficulties have been reported in reproducing consistent mortality experimentally with *E. rhusiopathiae* isolates of avian origin (3, 32). Reducing the number of passages on artificial media to an absolute minimum appears essential to maintaining virulence of the organism. Boyer and Brown (14) maintained virulence of an *E. rhusiopathiae* strain by storing infected liver at 4°C, which served as a source of the organism for further bird passage.

Sadler and Corstvet (95) and Corstvet *et al.* (28) showed that a few turkeys infected SC remained carriers for variable periods. Asymptomatic carriers of *E. rhusiopathiae* could not be detected before or after necropsy. The number of organisms used to challenge turkeys, route of inoculation (oral or parenteral), administration of antibiotics, time after vaccination and challenge, and age of the turkey apparently did not influence this carrier state, which is produced in very few birds. Isolations of *E. rhusiopathiae* from carriers are most frequent from cecal tonsils, liver, large intestine, heart, and blood (26, 28, 95). Xu *et al.* (143) reported a total of 95 isolates obtained from the pharynx of healthy chickens, ducks, and geese. Corstvet (25) found the organism shed in feces of a few birds up to 41 days postinoculation. In other experiments, the organism was found to persist in blood for several weeks postinoculation (28).

The role of vectors in transmission of erysipelas is still unclear. Wellmann (133) found that this pathogen could be transmitted mechanically from sick mice to pigeons by the stable fly, horsefly, mosquito, and other biting flies. Recently, Chirico *et al.* (21) identified that the poultry red mite, *Dermanyssus gallinae*, can act as a reservoir for *E. rhusiopathiae. E. rhusiopathiae* was isolated from the integument as well as the interior of the mite.

Incubation Period

In naturally occurring outbreaks, the incubation period cannot be readily ascertained. Experimental SC inoculation of turkeys with 10^4–10^6 organisms usually kills most of them in 44–70 hours; a few may die after 96–120 hours. With oral exposure, signs of disease generally occur 2–3 days later than with SC inoculation, and with a lower death rate. Occasionally, a turkey dies 2–3 weeks after oral exposure. A SC inoculum of 10^2, instead of 10^4 or 10^6, organisms delays clinical signs by about 24 hours. The incubation period does not seem to vary among turkeys 7, 12, 16, and 20 weeks of age or between sexes.

Clinical Signs

In turkey flocks, outbreaks usually start suddenly, with losses of one to several birds; owners may suspect that deaths are from poisoning, stampede injuries, or predators. A few droopy birds (especially toms) may be noticed, but these individuals are usually easily aroused. Just prior to death, some birds may be very droopy, with unsteady gait. Some may have cutaneous lesions; affected males may have swollen purplish, turgid snoods (fleshy tubular appendage on dorsal surface of head). Some turkeys are now "desnooded" shortly after hatching; as a result, such lesions are infrequently seen. Gradual emaciation, weakness, and signs of anemia occur in some cases in which endocarditis is the cause of death; other turkeys with vegetations (especially vaccinated birds) may die suddenly without signs, probably as a result of emboli. Sudden losses of hens 4–5 days after artificial insemination with peritonitis, perineal congestion, and skin discoloration have been reported.

Main clinical signs in chickens are general weakness, depression, diarrhea, and sudden death. In laying chickens, egg production may be decreased. Kilian *et al.* (60) reported no immediate drop in egg production in laying pullets, although signs were evident; later, there was about a 50–70% drop. Affected ducks, geese, pheasant, and quail generally are depressed, have diarrhea, and die suddenly.

Morbidity and Mortality

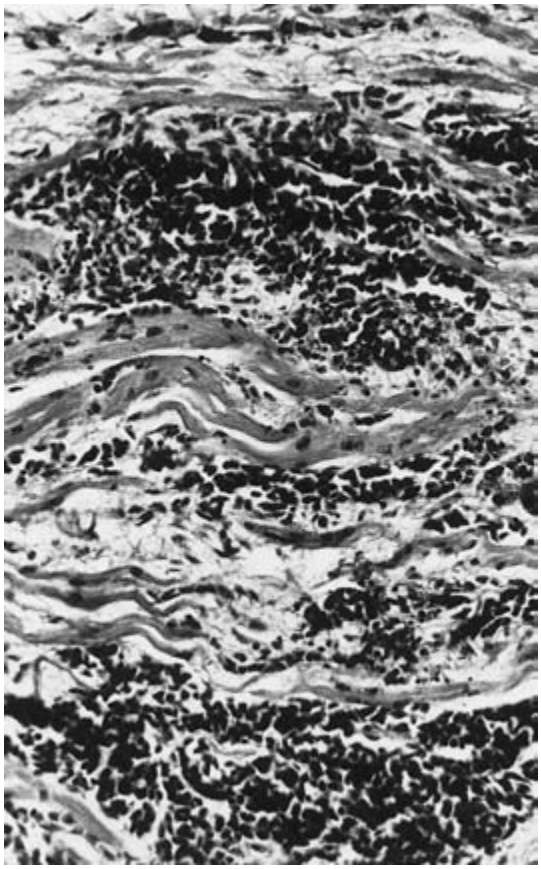
Mortality rates vary among different avian species. Morbidity and mortality are frequently about the same in unvaccinated flocks of turkeys and other species of poultry because most sick birds die. These parameters are also dependent on whether prior immunization or early treatment was administered. In immunized flocks some birds may be depressed and recover. The pattern of morbidity and mortality may range from sickness or death of occasional birds in good condition to sudden loss of several within 24–48 hours. Mortality can range from much less than 1% to as high as 25–50% of a given group, although adjacent groups may not be affected.

Pathology

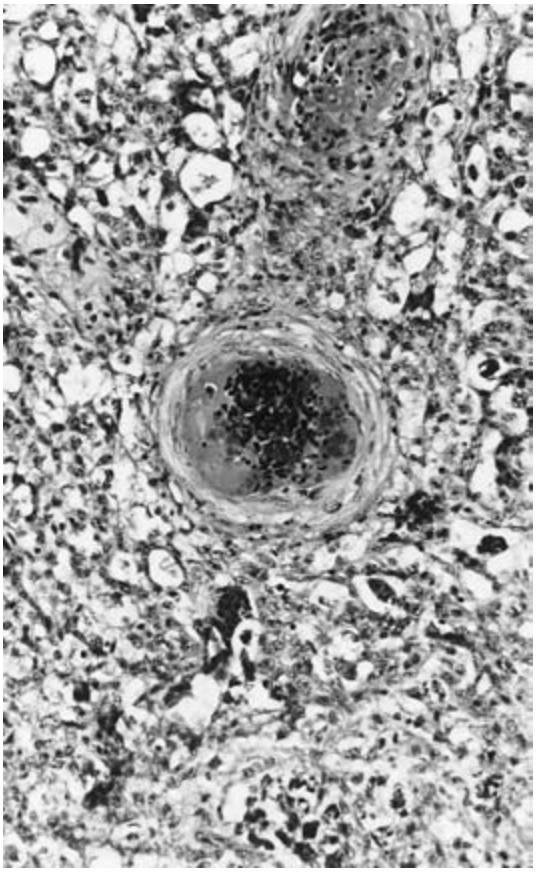
Gross

In naturally occurring outbreaks, the lesions are suggestive of a generalized septicemia, and the following lesions have been observed in different outbreaks in turkeys. There is generalized congestion; degeneration of fat on the anterior edge of the thigh; degeneration and hemorrhage in pericardial fat; petechial hemorrhage in abdominal fat; hemorrhage in heart muscle; and a friable, enlarged, and possibly mottled liver, spleen, and usually kidney. Other gross lesions may be fibrinopurulent exudate in joints and pericardial sac, fibrin plaques on heart muscle, thickening of the proventriculus and gizzard wall with ulceration, small yellow nodules in ceca, catarrhal or sanguinocatarrhal enteritis, vegetative endocarditis, dark crusty skin lesions, a turgid irregular reddish purple snood in toms, hydropericardium, and distended visceral blood vessels (93). Other lesions noted with varying frequency in field outbreaks were diffuse skin reddening and a dirty brick-red muscle color. Some birds that died had no lesions other than slight catarrhal enteritis and petechiae in the heart fat.

In experimental infections, some of the lesions observed in naturally occurring outbreaks are not uncommon. Endocarditis, except in vaccinated birds, is rare in experimentally infected birds. In some field cases, and in birds vaccinated twice or more with bacterin and intravenously challenged, congestive heart fail-



23.9. Acute erysipelas, turkey heart. Interstitial hemorrhages and separation of myocardial fibers (edema). H & E, ×100.



23.10. Acute erysipelas, turkey liver. Fibrin thrombus containing bacterial aggregates in the central portal blood vessel. Severe vacuolar degeneration of surrounding hepatocytes and several large basophilic sinusoidal, reticuloendothelial (Kupffer) cells are evident. H & E, ×100.

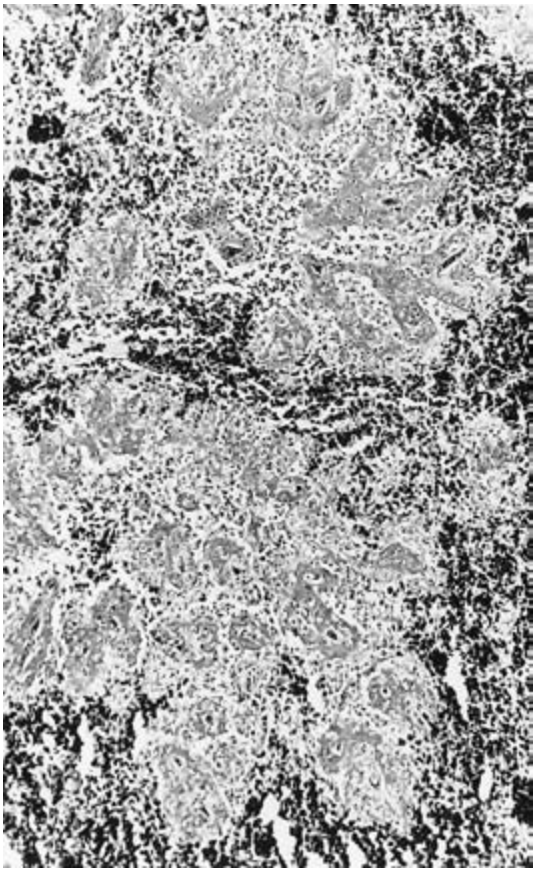
ure with vegetations of the atrioventricular valves (sometimes extending as much as 7 cm into the aorta) have been found. Asymptomatic carrier turkeys are usually free of gross lesions.

In at least 2 field outbreaks in chicken layers, lesions in endocardium, joints, and skin were not observed (10). In ducks, geese, and pheasant, lesions are similar to those observed in other avian species, with the addition of dark congested areas in foot webs of ducks.

Microscopic

Generally, the histopathologic features of acute erysipelas in turkeys reflect rather closely the observed gross findings, and specific cellular alterations are those expected in septicemic infections (9). Vascular changes dominate the histopathologic picture, with generalized engorgement of blood vessels and sinusoidal channels in virtually all organs. Although there may be a strong central (cardiac or vasomotor) basis for vascular congestion, there is also evidence of direct vascular damage. Intravascular aggregations of bacteria accompanied by fibrin thrombi are frequent in capillaries, sinusoids, and venules. Overt hyalinization of walls of affected vessels is not uncommon. Edema and hemorrhage, especially prominent in lung and heart (Fig. 23.9), are further evidence of severe vascular damage. In addition, rounding up of vascular endothelial cells or mononuclear phagocytic system (MPS) cells of sinusoids is a consistent histologic finding, and engulfed bacteria are demonstrated readily in MPS cells in liver and spleen.

Damage to parenchymal cells is generalized in acute erysipelas and is especially obvious in liver, spleen, and kidney. Degenerative changes in hepatic cells vary from cloudy swelling with cellular dissociation to overt coagulative necrosis. Focal or massive necrosis apparently related to thrombosis of major portal vessels is seen occasionally, but diffuse degenerative changes are much more common (Fig. 23.10). In spleen, the earliest observable change is necrosis and lysis of lymphoid elements. This progresses to nearly total loss of lymphocytes, with the hyalinization of sheathed arteries of white pulp and surrounding reticular elements (Fig. 23.11). The epithelium of proximal tubules undergoes early degenerative change in affected turkeys. Swelling, dissociation, and separation from the basement membrane are frequent and prominent changes that are seen in renal epithelial cells; overt coagulative necrosis is rare. Certain of these pathologic changes in kidneys have also been reported by others (127). It is not unusual to find degenerative changes or necrosis in a variety of other organs such as lung, heart, pancreas, gastrointesti-



23.11. Acute erysipelas, turkey spleen. Hyalinized sheathed arteries within 2 malpighian corpuscles and nearly total depletion of lymphocytes are evident. Surrounding sinusoids are engorged with erythrocytes. H & E, ×100.

nal tract, skeletal muscle, and skin. These changes are, however, seldom as remarkable in frequency or magnitude as parenchymatous changes in liver, spleen, and kidney. Regardless of the site of involvement, hemorrhage and fibrin deposition frequently accompany parenchymatous necrosis.

The cellular inflammatory component of peracute or acute erysipelas lesions is minimal. In scarified skin of turkeys infected by this route, there may be extensive heterophil infiltration, congestion, edema, and necrosis. Cellular inflammatory response as judged from examination of field cases is more prominent in turkeys with subacute or chronic disease. Heterophil and mononuclear leukocytic infiltration as well as proliferation of MPS cells can be found around necrotizing lesions in liver and spleen as well as in heart valves and synovial membranes of joints. The pathology of experimentally induced subacute or chronic erysipelas in turkeys has not been reported.

In a study of acute erysipelas outbreaks in chickens (10), histopathologic alterations very similar to those described in turkeys were reported. Shibatani *et al.* (104) described disseminated intravascular coagulation in chickens inoculated experimentally. These investigators also observed depletion of lymphocytes in the bursa of Fabricius and thymus and suggested that thromboplastin released from these tissues following depletion played a role in disseminated intravascular coagulation.

Pathogenesis of the Infectious Process

The precise mechanism(s) by which the organism causes disease is still not very well understood. *E. rhusiopathiae* appears to be able to gain access to the body by the oral route or by entry through breaks in the skin. In swine, *E. rhusiopathiae* can induce disease by gaining access through the palatine tonsils or other pharyngeal lymphoid tissue. This mode of pathogenesis is probable for turkeys based on experimental evidence with orally administered challenge cultures (25) and live vaccines (16).

Although not completely clear, the pathogenicity of different isolates of *E. rhusiopathiae* may be related to their ability to produce various virulence factors to help them to resist or survive phagocytosis and to avoid host defenses. These factors include specific attachment factors (capsule or adhesins), enzymes such as neuraminidase and coagulase, and stress proteins. Takahashi *et al.* (117) reported a correlation between pathogenicity for mice and swine and the ability of *E. rhusiopathiae* to adhere to porcine kidney cells. Other investigators have demonstrated that *E. rhusiopathiae* isolated from swine with endocarditis showed a higher degree of adherence to porcine heart valve tissue (15, 63). Recently, Shimoji *et al.* (106) identified two adhesive surface proteins designated RspA and RspB that were able to bind to fibronectin and type I and IV collagens. Partridge *et al.* (87) suggested the expression of high levels of a stress protein could allow the organism to resist oxidation and survive the low pH environment of the phagolysosome following phagocytosis. The production of coagulase may allow *E. rhusiopathiae* to wall itself off from host defenses. More studies are needed to fully elucidate the pathogenesis of *E. rhusiopathiae* in avian species.

Immunity

Active

Birds recovered from acute infections have a high degree of resistance to reinfection and death. Killed bacterins, the current commercially available immunoprophylactic agent for the immunization of turkeys against erysipelas, will prevent disease under both experimental and field conditions. Bacterin in conjunction with penicillin at the beginning of an outbreak will usually control losses. Long-lasting immunity is not produced by only one bacterin injection in turkeys. The immune response is more effective from 2 or more doses at intervals of at least 2–4 weeks. Experiments with 4–7-week-old turkeys have shown that protective immunity begins to decline between 4 and 5 weeks post-vaccination. Krasnodebska-Depta and Janowska (64) reported that 28- and 30-week-old turkeys vaccinated with a bacterin made for swine were resistant to challenge 2 months post-vaccination. Aerosol immunization of ducks with an erysipelas vaccine has been reported to be very effective in eliminating the disease.

Osebold *et al.* (85) reported limited protection with a live vaccine strain that they administered SC to turkeys followed by subsequent challenge with a virulent strain. Bricker and Saif (16) reported on an effective level of protection in turkeys vaccinated

through the drinking water with a live serotype 1a strain of *E. rhusiopathiae,* followed by challenge with the homologous serotype. Two vaccine doses administered 2–3 weeks apart were necessary to induce a sufficient protective response that lasted at least 3 weeks following administration of the second vaccine dose. Administration of this live vaccine directly into the esophagus of turkeys did not induce a protective immune response. It is speculated that lymphoid tissue in the pharyngeal region plays a role in the induction of an immune response to organisms introduced by the drinking water route.

Passive

Treatment with swine erysipelas antiserum (horse origin) alone is said to be of some value if administered very early, but it is not practical because of the expense and the lack of uniform efficacy. Antiserum and penicillin have been used successfully in ducks (91).

Diagnosis

Isolation and Identification of Causative Agent

Because gross lesions of dead birds indicate septicemia, diagnosis depends on the demonstration and identification of *E. rhusiopathiae.* Rapid presumptive diagnosis is provided by the presence in liver, spleen, heart blood, or bone marrow smears of clumps and segregated gram-positive, beaded, slender, and pleomorphic rods. Particularly helpful with decomposed specimens are bone marrow culture and smear. Viemmas *et al.* (131) described the use of immunoperoxidase staining to detect *E. rhusiopathiae* from the tissues of experimentally infected turkeys.

Isolation of *E. rhusiopathiae* from sick birds that are killed and then cultured is neither as easy nor as frequently positive as culturing from birds dead of the disease. Detecting the organism from a carrier requires multiple samples from various tissues. Isolation from endocardial tissues is facilitated by finely mincing before inoculating enrichment broth. With birds dead from the infection, however, liver, spleen, or bone marrow will suffice. Useful inhibitory media are the sodium azide-crystal violet medium described by Packer (86) and the tryptose phosphate broth medium with 5% horse serum plus kanamycin, neomycin, vancomycin, and novobiocin described by Wood (137). These media are the most satisfactory, although they do not completely prevent growth of other organisms (particularly with samples of intestinal contents) and may inhibit growth of some strains of *E. rhusiopathiae* (15). Shimoji *et al.* (109) described a selective media containing tryptic soy broth, ethidium bromide, and sodium azide. For primary isolation, recovery is favored by inoculating biplates containing 5% blood agar and Packer's medium followed by incubation in an atmosphere of 5–10% CO_2 or reduced oxygen. Ordinary atmosphere is suitable after a few passages on artificial media. Typical colonies composed of gram-positive rods are selected and placed in TSI medium or Kligler's lead acetate medium and incubated for 24 hours at 37°C. An excellent presumptive test for *E. rhusiopathiae* is a blackening of the medium (due to H_2S production) before there is a very noticeable change in the medium color.

Makino *et al.* (68) described a direct, rapid, sensitive test for detection of both *E. rhusiopathiae* and *E. tonsillarum* using the polymerase chain reaction methodology. Using primers to amplify a 937-bp DNA region from *E. rhusiopathiae*, Shimoji *et al.* (109) designed a broth cultivation-PCR technique for rapid diagnosis of erysipelas. In a report comparing detection methods, Fidalgo *et al.* (38) indicated that a combination of cultural and PCR methodologies was optimal. At 24 and 48 hours of broth incubation, PCR was performed with primers ER1 and ER2 (109). Takeshi *et al.* (123) performed a PCR test using 4 specific primer sets for the identification of *E. rhusiopathiae, E. tonsillarum*, and strains representing serotypes 13 and 18. Hennig *et al.* (49) used DNA obtained from histochemically processed liver tissue to diagnose erysipelas in ring-necked pheasants by the PCR technique.

Though rarely used in diagnostic laboratories because of newer molecular-based methodologies, the mouse may be used for a confirmatory protection test using erysipelas antiserum (the *E. rhusiopathiae* isolate, however, must be pathogenic for mice). One group of experimental animals is inoculated parenterally with a 24-hour culture of the isolate; another is inoculated with *E. rhusiopathiae* antiserum and immediately thereafter with the isolate. The unprotected group should die within 4 days, whereas animals receiving antiserum will live. Cooper and Bickford (23) suggested the use of a mouse ear scarification model for isolating *E. rhusiopathiae* from a mixed culture. Briefly, 24-, 48-, and 96-hour broth cultures are applied to the scarified ears of several mice using a cotton swab. The mice usually die within 3–6 days, and the organism can be isolated in pure culture from the liver or heart blood. *E. rhusiopathiae* may be detected in tissues using the fluorescent antibody technique (73, 81), however, specific fluorescein-labeled antibody is not commercially available.

Sudden losses of adolescent turkeys in good flesh but with septicemic lesions, IM and subpleural ecchymotic and suffusion hemorrhages, and erysipeloid swelling of the snood suggest erysipelas. Also significant is a marked hemorrhagic condition of the skin, facial, and muscular tissues of the breast. In many cases, predominant losses will be in males, but in breeding flocks, sudden losses in hens with peritonitis, SC, cutaneous discoloration, and a history of insemination just prior to this suggest *E. rhusiopathiae* infection. Diagnosis is further validated by procedures described previously and in more detail by Cooper and Bickford (23).

Serology

Plate, tube, and microagglutination tests, in addition to passive hemagglutination, hemagglutination-inhibition, complement-fixation, growth agglutination, and growth-inhibition tests, have been used in swine erysipelas research, but their usefulness for the diagnosis of avian erysipelas have not been evaluated fully. Actual information regarding the nature of immunity induced in turkeys following natural exposure to *E. rhusiopathiae* or to erysipelas vaccines is scarce. Infected birds that recover appear to be solidly immune. Agglutination titers have been reported to be usually 160 or higher in antibiotic-treated recovered turkeys and turkeys that are carriers for the organism. Sikes and Tumlin

(113), however, suggested that titers of 40 or greater are indicative of *E. rhusiopathiae* infection in turkeys. Takahashi *et al.* (122) reported positive growth agglutination test titers in culled chicken layers sent to a processing plant. Currently, it is not known whether one particular serotype or strain of the organism is effective in detecting antibodies to other serologic types or strains of *E. rhusiopathiae*. Sato *et al.* (99) described latex agglutination and ELISA tests using the 64,000-MW protective antigen of *E. rhusiopathiae.* At present, these tests are mainly useful for research studies.

Differential Diagnosis

Fowl cholera, *Escherichia coli* infections, salmonellosis, and peracute Newcastle disease might be confused with the acute septicemic form of the disease. The less common forms of the disease (urticaria and endocarditis) may be caused by other miscellaneous bacterial agents or possibly fungal pathogens. All of these agents are differentiated easily from *E. rhusiopathiae* by gram staining or biochemical activity. *Lactobacillus* sp., which occasionally may be isolated from intestinal tracts or livers of poultry and is biochemically similar to *Erysipelothrix*, may be differentiated using the highly selective Packer's medium containing sodium azide and crystal violet. Gram staining, growth on Packer's medium, and the typical reaction pattern in TSI medium or Kligler's lead acetate medium are excellent presumptive tests. Confirmatory diagnosis is by molecular methodologies such as PCR, fluorescent antibody test, or animal pathogenicity tests.

Intervention Strategies

Management Procedures

It has been suggested, though not established, that various environmental factors may make avian species more susceptible to *E. rhusiopathiae* infection. Early field observations indicated that the beginning of rainy, cold weather, which frequently coincided with sexual maturity, was related to outbreaks of erysipelas in turkeys on the range. This may apply to flocks of other avian species. Source of the organism may be contaminated feed, soil, or decaying matter; infected carrier birds in the flock; or infected rodents. Apparently, the relationship between environmental factors and disease does not apply to cases involving individually confined birds, as in a zoo.

It is not possible to make clear-cut and specific recommendations for preventive or control management. A general suggestion is to use clean disinfected equipment and to rotate turkey ranges away from previously contaminated areas. Certain disinfectants, notably 1–2% sodium hydroxide (lye) solutions, are effective against *E. rhusiopathiae*; phenols, cresols, and related disinfectants; iodine; and certain household soaps are moderately effective.

Beneficial management practices in handling an outbreak of erysipelas in turkeys include thorough decontamination of equipment, prompt removal of dead birds and other carrion from premises, encouraging adequate feed and water intake, and handling birds as little and as gently as possible or practical. If unlimited range is available, it might be desirable to move the flock to clean ground, but such a practice may contaminate the new range.

With no specific and effective recommendations for management control of this disease in turkeys, it is suggested that birds be properly immunized in areas where erysipelas is known to be a problem.

Vaccination

Types of Vaccine

The most widely used products for the immunization of turkeys against erysipelas are the formalin-inactivated, aluminum hydroxide-adsorbed *E. rhusiopathiae* bacterins which may contain either whole or lysed bacterial cells. These bacterins initially were developed for use in swine and were shown to be effective in preventing erysipelas in turkeys (2, 3, 24). With the characterization of the 64,000-MW protective antigen, several investigators have suggested substitution of nonanimal evaluations, such as antigen-specific ELISA, for the routine release of these products (7, 47, 48).

Only certain strains of *E. rhusiopathiae* belonging to serotype 2, however, have been effective for use in bacterins. These strains are highly immunogenic because of the production of a soluble immunizing substance. This soluble immunizing substance is adsorbed and precipitated by aluminum hydroxide, which also adsorbs either the whole or lysed bacterial cells. The presence of this soluble substance is considered necessary for the production of an effective bacterin.

Although live erysipelas vaccines for swine have been around for more than 40 years, only recently have live vaccines become available for use in turkeys. Live erysipelas vaccines induce cross-protection against heterologous serotypes (90).

Other more recent approaches to vaccine development have focused on acapsular mutants, truncated protective antigen and the use of bacterial vectors for expressing *E. rhusiopathiae* protective antigen (20,54,110).

Field Vaccination Protocols and Regimes

A regimen of immunization can be suggested for meat turkeys as well as those kept for hatching egg production. Because the disease in other avian species is so sporadic, immunization other than in turkeys is not generally recommended. It is also important to remember that effective immunization of mice or swine is not an adequate demonstration of the ability of a bacterin to protect turkeys. Cultures avirulent for turkeys and without immunogenicity for them may kill mice or provide protection. At this time, immunizing capacity for turkeys can be assessed properly only with challenge of vaccinated turkeys.

Suggested for meat turkeys in areas of high risk is a single dose of the bacterin inoculated SC at the dorsal surface of the neck behind the atlas. The original investigation and demonstration of efficacy were based on IM injection of bacterin; however, because of the possibility of sterile abscesses (with downgrading at slaughter), SC inoculation is used now.

For turkeys kept as breeders, at least 2 doses of bacterin given at 4-week interval, should be administered prior to onset of egg production. The first dose may be given at 16–20 weeks of age (at selection time) and an additional dose given (2 mL/hen, 4 mL/tom) just prior to beginning of lay.

Improved vaccines properly used, adequate testing of the biologicals, planned immunization programs based on flock and premise history, and proper diagnosis of disease outbreaks as a basis for prompt treatment must be combined for effective prevention of erysipelas.

Treatment

The antibiotic of choice for an outbreak in market or breeder turkeys is a rapid-acting form of penicillin. Any antibiotic should be used under veterinary supervision according to current treatment procedure. Label directions should be followed carefully. As soon as diagnosis is definitely established, potassium or sodium penicillin should be administered IM at the rate of about 10,000 U/lb body weight (200,000–300,000 U) simultaneously with a full dose of erysipelas bacterin. Control usually has been attained by giving penicillin (1,000,000 U/gal) in drinking water for 4 or 5 days to all birds in the flock when a presumptive diagnosis is made. All birds in the affected flock should be treated. Some recommendations suggest the use of procaine penicillin or other longer-acting derivatives; under certain circumstances, these may be used successfully, but in outbreaks, rapid-acting formulations are almost mandatory. A combination of longer-acting and rapid-acting antibiotic formulations may provide best control if individual SC or IM injection is feasible and cost effective. Especially in meat-bird flocks, however, catching and handling each bird may be impractical or even harmful. Sterile abscesses and downgrading may follow IM injections. Care should be taken to observe the required withdrawal periods for the antibiotic used. Turkeys and possibly other birds with advanced signs of the disease at treatment time often will not recover. Experimental use of certain antibiotics including penicillin will control the infection but will not eliminate carriers. Antibiotics other than penicillin may increase the carrier rate in a flock of turkeys (27).

Comparative studies are lacking on efficacy of various antibiotics for controlling *E. rhusiopathiae* infection in avian populations. Erythromycin and broad-spectrum antibiotics have been found effective. *In vitro* studies have shown the organism to be resistant to neomycin (39). Sulfonamides and oral oxytetracycline are not effective treatments.

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Avian Intestinal Spirochetosis

David J. Hampson and David E. Swayne

Introduction

Avian intestinal spirochetosis (AIS) is a disease of birds characterized by a pronounced colonization of the cecum and/or rectum with anaerobic intestinal spirochetal bacteria of the genus *Brachyspira*. AIS has been recorded mainly in flocks of laying hens and broiler (meat) breeder hens, where it causes mild to moderate, subacute to chronic disease (26, 32, 100, 142, 155, 164). AIS has not been diagnosed in broiler flocks, but has been reported as a sporadic condition in other domesticated poultry such as turkeys (131) and game birds including pheasants and partridges (73, 177). Severe AIS has been reported in common rheas (*Rhea americana*) (130, 161) and in geese (110). Subclinical colonization either with pathogenic or apathogenic *Brachyspira* species is common in feral waterfowl, particularly ducks (74, 117).

The clinical outcome following a given exposure depends on the age and species of bird involved, the pathogenic potential of the particular *Brachyspira* species or strain, the extent of colonization, and likely many other predisposing or mitigating factors. The most commonly reported clinical problems in chickens with AIS are delays or reductions in egg production, smaller and lighter eggs with poor shell quality, and a change in fecal consistency, with increased fecal output and water content (26, 36, 46, 51, 144, 155, 164). Diarrhea may be yellowish brown, mucoid and/or foamy, with increased lipid content (36, 155, 164). These changes result in fecal staining of eggshells and housing problems including contamination of cages and equipment, wet litter, reduced hygiene, increased odor and attraction of flies. Reductions in growth rate in broiler chicks hatched from infected breeding hens also have been reported (37, 132).

The spirochetes involved in AIS are distinct from *Borrelia anserina*, the etiological agent of non-relapsing, tick-borne, acute septicemic borreliosis (sometimes called avian spirochetosis) (see "Miscellaneous and Sporadic Bacterial Infections" later in this chapter). The spirochete genus *Brachyspira* currently includes seven officially named and two unofficially proposed species of intestinal spirochetes (31, 49, 65, 115, 138, 142). These nine *Brachyspira* species colonize the large intestine of various mammalian hosts, and all but two of the species have been recorded in birds. Other uncharacterized, uncultured, and probably commensal spirochete species also exist in the large intestines of birds and mammals (52).

Certain of the *Brachyspira* species form part of the normal large intestinal microbiota whilst to a greater or lesser extent others act as pathogens in individual avian or animal hosts. The four main pathogenic species found in birds are *B. intermedia, B. pilosicoli, B. alvinipulli* and *B. hyodysenteriae*. The best characterized of these is *B. hyodysenteriae*, the agent of swine dysentery

(56). Besides swine, *B. hyodysenteriae* naturally infects the common rhea, causing a severe necrotizing typhlitis (16, 17, 77, 130). *B. intermedia* is an enteric pathogen in chickens, but also is found in swine in which it sometimes may be mildly pathogenic (58). *B. pilosicoli* colonizes and is considered a pathogen in a range of avian and mammalian species, including swine, dogs, horses and humans (30, 31, 48, 57, 58, 171). *B. alvinipulli* has only been reported as a pathogen in chickens and geese (110, 155, 157).

The clinical signs of AIS are not specific, and accurate diagnosis largely relies on identification of pathogenic *Brachyspira* species in the ceca and rectum of affected birds. These spirochetes are slow growing anaerobes, requiring specialized media for isolation, and consequently only laboratories with expertise in their culture and identification are able to routinely confirm a diagnosis. In part this helps explains why AIS is relatively infrequently diagnosed, despite epidemiologic surveys identifying it as being common in laying hen and broiler breeder flocks (8, 26, 32, 100, 142).

A recent review of AIS has been published (143). A text is available on intestinal spirochete infections of domestic animals and humans (52), and this includes a chapter on AIS (154).

Definitions and Synonyms

Avian intestinal spirochetosis is a general term referring to colonization of the avian cecum and/or rectum with spirochetes. More specifically, the term is used to describe colonization with pathogenic *Brachyspira* species (*B. intermedia, B. pilosicoli* and/or *B. alvinipulli*) in flocks of chickens or other birds with reduced egg production and/or diarrhea, and for the severe typhlitis of rheas caused by *B. hyodysenteriae.*

The related term "intestinal spirochetosis" is used to describe colonization of humans with either or both of the two *Brachyspira* species *B. pilosicoli* and *B. aalborgi* (47, 103, 166). "Porcine intestinal spirochetosis" or "porcine colonic spirochetosis" is used to describe infections of swine with *B. pilosicoli* (48), whilst the name "swine dysentery" is reserved for infections of swine with *B. hyodysenteriae* (56). In the future, it may be desirable to further subdivide or refine the term AIS to link it with specific etiologic spirochete species or clinical outcomes.

Economic Significance

A recent study in the United Kingdom (UK) has suggested that AIS caused by *B. pilosicoli* results in a potential annual loss to the laying hen industry of around £4.1 million (US$ 7.6 million) (20). Inadvertantly a sum of £14 million was reported in the publication, but later it was realized that this did not take into account the prevalence of *B. pilosicoli* infections (~30%) (18). The £14 million was probably about right, however, as the calculation also did not include losses associated with *B. intermedia* infections; the latter are often more severe than *B. pilosicoli* infections, and are commonly found in noncaged (free-range and barn) flocks that account for approximately 50% of UK production (18). Losses similar to those in the UK likely occur elsewhere, as, for example, epidemiologic surveys suggest that AIS is as common amongst Australian and Italian laying hen flocks as it is in the UK (8, 100, 142).

Worldwide losses to the meat chicken industry associated with infection of breeder flocks also may be high, especially as these flocks are commonly infected (100, 142). In 1998 it was calculated that a commercial broiler flock hatched from eggs from a breeder flock with clinical AIS lost approximately £9,900 (US$ 15,800) per annum from reduced growth rates and poor feed digestion (132). Additional losses to the infected breeder flocks associated with reduced egg production and increased feed consumption were estimated at £10,600 (US$ 16,900) per flock per annum.

Besides delayed and/or reduced egg production in laying hen flocks, AIS can reduce profitability due to mortalities, downgrading of stained eggs, and increased labor costs associated with extra cleaning of cages and houses. There also may be local adverse environmental effects through increased odor from wet feces and attraction of flies.

Public Health Significance

Certain avian strains of *B. pilosicoli* are closely related to strains from humans (and other animals), and there probably is no barrier to cross-species transmission of this spirochete species (58). Strains of *B. pilosicoli* isolated from humans have been used experimentally to colonize 1-day-old chicks (34, 106, 167, 168) and adult laying hens (72), and there seems to be no reason why avian strains could not colonize humans. Nevertheless, it remains unproven whether transmission occurs from birds to humans. Colonization of people with *B. pilosicoli* is common in individuals in developing countries, whilst in developed countries it is mostly confined to homosexual males and those with suppressed immune systems (10–50% prevalence) (98, 103, 108, 166, 171). In humans, colonization with *B. pilosicoli* has been linked to problems such as abdominal pain, chronic diarrhea, reduced growth rates in children (14, 29, 47, 103, 121), and spirochetemia (81, 172). Infection is common in individuals in crowded and/or unhygienic conditions, and its occurrence has been linked with certain sources of drinking water (98, 108). *B. pilosicoli* has been isolated from lake and dam water frequented by ducks colonized by *B. pilosicoli*, emphasizing the potential for some avian intestinal spirochetes to be transmitted by ingestion of contaminated water (117). The likelihood of healthy poultry industry workers becoming infected with *B. pilosicoli* from contact with chickens is low.

History

There are several early accounts of intestinal spirochetes in birds. For example, in 1910 an intestinal spirochete called "*Spirochaeta lovati*" was described in the ceca of normal young and adult grouse (39). In 1930, helically shaped bacteria of three morphologic types were visualized in cecal droppings from both clinically normal and sick chickens obtained from Baltimore live-poultry markets (62). In 1955, large caseous nodules with associated spirochetes were identified in cecal walls of turkeys, chickens, and pheasants in the United States of America (US) (99). In the early 1970s a renewed interest in intestinal spirochetes was sparked by the discovery that the spirochete *Treponema hyodysenteriae* (later called *Serpulina hyodysenteriae* and now *Brachyspira hyodysenteriae*) was the etiologic agent

of swine dysentery, an important mucohemorrhagic diarrheal disease of pigs (56, 63, 158).

In the mid to late 1980s, a series of studies conducted mainly in the Netherlands (26, 32–37), but also in the UK (46), identified intestinal spirochete infections as being common in flocks of laying hens and in broiler breeder hens, in which they were associated with a variety of syndromes including delayed and/or reduced egg production and wet feces. At that time the species of spirochete involved were not known. Subsequent work in the US (155, 164), and more recently in Australia (100, 125, 144, 146) and Europe (8, 19, 20) has confirmed and extended these results, including the identification and naming of the pathogenic *Brachyspira* species causing disease in poultry (101). In 1990, AIS with necrotizing typhlitis and high mortality was identified in common rheas in the US (130). In recent years sporadic cases of AIS also have been identified in domestic turkeys (131), pheasants (177), partridges (73) and geese (110).

Etiology

Classification and Host Specificity

Spirochetes are classified into the order Spirochaetales in the three families Spirochaetaceae, Brachyspiraceae, and Leptospiraceae, and in 9 genera (21, 119). The intestinal spirochetes associated with clinical cases of AIS all belong in the family Brachyspiraceae, genus *Brachyspira* (119).

The genus *Brachyspira* currently contains seven official species (*B. hyodysenteriae, B. intermedia, B. innocens, B. murdochii, B. alvinipulli, B. pilosicoli and B. aalborgi*), and two proposed species ("*B. pulli*" and "*B. canis*") (31, 49, 65, 115, 135, 137, 138, 142, 143, 170). Most of these species were originally delineated using multilocus enzyme electrophoresis (MLEE) (91, 94, 101), coupled with analysis of their 16S ribosomal RNA gene sequences and phenotypic features (40, 120, 136). All species except *B. aalborgi* previously belonged to the genus *Serpulina* (49, 115). All the species colonize the large intestine, but only *B. pilosicoli* and *B. aalborgi* are known to attach by one of their cell ends to cecal or colonic enterocytes (103). The *Brachyspira* species have close similarities in their 16S rRNA gene sequences, indicating that they have evolved into different species relatively recently (118). Of the nine species, to date only *B. aalborgi* and "*B. canis*" have not been recovered from birds. The wide diversity of *Brachyspira* species found in birds suggests that they may have been the original hosts of an ancestral *Brachyspira*-like anaerobic spirochete when it first colonized the intestinal tract.

Recently, unusual spirochetes apparently of the genus *Brachyspira* have been identified in jackdaws, hooded crows and Rooks (genus *Corvus*), although their significance is uncertain (75). Other unclassified avian spirochetes have been described that differ biochemically, morphologically, and/or genetically from the earlier described species (101, 147, 148, 169).

Pathotypes

Based on experimental studies and naturally occurring colonization, the species *B. intermedia, B. pilosicoli, B. alvinipulli* and *B. hyodysenteriae* are considered to be potential pathogens in birds and have all been used experimentally to reproduce disease. Their features are summarized in Table 23.4 (51, 101, 144, 157). The proposed species "*B. pulli*", together with *B. innocens* and *B. murdochii* are generally thought of as being commensal in birds (101). Consistent with this, strains of *B. innocens* have failed to cause disease in experimentally inoculated chicks and breeder hens (144, 167). On the other hand, a strain of "*B. pulli*" was mildly pathogenic in experimentally inoculated chicks (50), and strains of *B. murdochii* were present in many hens from a broiler breeder flock with AIS (142, 146).

Besides the spirochete species, expression of pathogenicity varies with route of inoculation, age of host, host species, environmental stressors, diet, and the presence of certain bacterial species in the large intestinal microbiota (152, 155).

Morphology and Staining

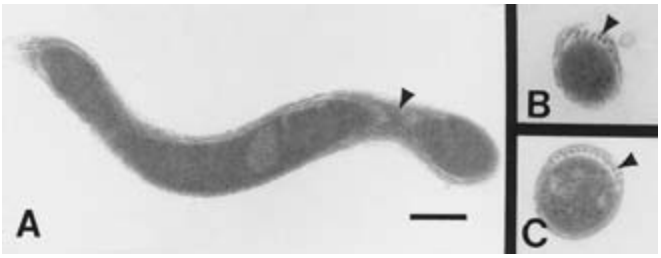
Brachyspira species are Gram-negative, helically shaped bacteria with diameters ranging from 0.25–0.6 μm, lengths from 3–19 μm, amplitudes from 0.45–0.79 μm, and wavelengths from 2.7–3.7 μm (63, 135, 137, 138, 170). They stain brown with silver-impregnation stains, blue with Wright's-Giemsa stains and are identified readily in wet mounts by dark-field or phase contrast microscopy. Each spirochete cell contains a central protoplasmic cylinder, multiple periplasmic flagella, and an outer envelope (outer sheath) (Fig. 23.12) (21). The periplasmic flagella are endocellular and divided into 2 equal sets, with each set originating from opposite poles of the protoplasmic cylinder and overlapping with the other set in the middle of the cell. This unique anatomic feature has been used as a phenotypic trait for identifying spirochetes. The number of periplasmic flagella has been used for spirochete classification, however this has limited value as the numbers can vary between and within species. *B. pilosicoli* and *B. aalborgi* typically have four flagella at each cell end, whilst the other *Brachyspira* species have eight or more at each end. The rotation of periplasmic flagella between the outer membrane and protoplasmic cylinder confers a corkscrew-like movement to spirochete cells (10, 22). These morphologic features and motility permit spirochetes to traverse highly viscous liquids, such as mucus, which immobilize externally flagellated bacteria (22, 109).

Growth Requirements

Brachyspira species are anaerobic, although they will tolerate transient exposure to air (134). Primary isolation has been accomplished on various selective solid media systems used to isolate swine intestinal spirochetes (1, 76, 86). Typically, media contain a blood agar base, such as Trypticase Soy agar with 5–10% defibrinated sheep blood, and 1–5 selective antibiotics (including spectinomycin, rifampin, spiramycin, vancomycin, polymixin and/or colistin). As the different *Brachyspira* species vary in their tolerance to these antimicrobials, a recommended "general" *Brachyspira* plate contains 400 μg/ml spectinomycin and 25 μg/ml each of colistin and vancomycin (76). These antibiotics inhibit the growth of non-spirochetal enteric bacteria that would otherwise overgrow the slow-growing spirochetes. Incubation is

Table 23.4. Morphologic, biochemical, and other characteristics of the four main pathogenic *Brachyspira* species reported in birds.

Characteristic	Species			
	B. intermedia	***B. pilosicoli***	***B. alvinipulli***	***B. hyodysenteriae***
Birds most commonly affected	Layer and broiler breeder chickens	Layer and broiler breeder chickens; turkeys	Layer and broiler breeder chickens; geese	Common rhea
Pathogenicity	Moderate to mild	Moderate to mild	Moderate to mild (severe in geese)	Severe
Colonization of cecal epithelial surface	Random in lumen and crypts—not attached to epithelium	May be attached by one cell end to cecal enterocytes	Random in lumen and crypts—not attached to epithelium	Random in lumen and crypts—not attached to epithelium
ß-Hemolysis pattern	Weak (occasionally intermediate or strong)	Weak	Weak	Strong
Indole production	+	– (occasionally positive)	–	+ (Occasionally negative)
Hippurate hydrolysis	–	+ (occasionally negative)	+	–
Type strain	PWS/A^T – ATCC 51140	P43/6/78^T – ATCC 51139	C1^T – ATTC 51933	B78^T – ATCC 274164
References	49, 51, 137	115, 144, 170,	110, 138, 157,	16, 77, 115, 130



23.12. *Brachyspira alvinipulli.* Spirochete cell is helically shaped on longitudinal orientation (A). On transverse sections, an end (B) and the middle (C) of spirochete cells have 8 and 16 periplasmic flagella (arrows), respectively (157).

at 37–42°C for a minimum of 10 days; however, for most avian isolates visible growth is usually present in 2–5 days. Typical gaseous environments are 94% H_2 and 6% CO_2, generated using anaerobic gas packs in an anaerobic jar, or 80% N_2, 10% H_2 and 10% CO_2 in an anaerobic chamber (13). The spirochetes can be further propagated in anaerobic brain heart infusion broth containing 10% fetal bovine serum, with 1% oxygen to enhance growth (133), or in a pre-reduced anaerobic Trypticase Soy broth medium ("Kunkle's medium") (87). Growth of 10^8 to 10^9 cells per mL can be achieved in these broths within 2–3 days.

Colony Morphology and Hemolysis on Blood Agar

When inoculated onto agar, *Brachyspira* species grow as a dull flat sheen on the agar surface, forming confluent areas of growth with sharply defined edges, sometimes penetrating into the agar. With many isolates, growth on the agar is surrounded by a zone of hemolysis. Most of the *Brachyspira* species are weakly hemolytic, although *B. hyodysenteriae* (from pigs, rheas and ducks) is strongly hemolytic, and occasional avian strains of *B. intermedia* and other unidentified species can cause intermediate to strong hemolysis (73, 74, 100, 101). The presence of spirochetal growth should be confirmed by the characteristic morphology and motility of the bacteria on wet mounts using dark-field or phase contrast microscopy, or in fixed smears using Wright's-Giemsa or Gram's stain. Spirochetes stain weakly gram negative.

Biochemical Properties

Brachyspira species typically contain alkaline and acid phosphatase, esterase, esterase lipase, ß-galactosidase, and phosphorylase activities (147). Differences in hemolysis patterns, production of indole, hippurate hydrolysis and the presence or absence of ß-galactosidase and ß-glucosidase activities have been used to categorize isolates (40). A commercial API ZYM system and numerical 5-digit coding system for enzyme activities has been used in differentiating avian and mammalian isolates (68, 94). As these phenotypic properties can vary, more specific molecular based techniques now have largely replaced biochemical testing for species identification.

Susceptibility to Chemical and Physical Agents

Strains of *B. pilosicoli* have been shown to survive for extended periods in water, particularly at colder temperatures (4 days at 25°C; 66 days at 4°C)(117). Similarly, strains of *B. hyodysenteriae* and *B. pilosicoli* can survive for prolonged periods in porcine feces and in soil mixed with porcine feces (78–210 days at 10°C) (12). On the other hand, strains of *B. intermedia* and *B. pilosicoli* are relatively short lived in chicken feces (~3 days at 4°C, at 109/g feces), and do not persist in the environment of chicken houses (122). This reduced viability in chicken feces is likely due to its rather dry and acidic nature. Most common disinfectants are efficacious against *Brachyspira* species (122), although it is best first to remove organic matter (24). Cleaning, disinfection and resting of empty houses between batches of hens have the potential to break cycles of AIS on infected farms (122).

Virulence Factors

The mechanisms by which *Brachyspira* species cause disease in avian and mammalian species are incompletely understood. Development of AIS probably requires the activity of multiple virulence factors, and these may vary somewhat between the pathogenic species. In general terms, virulence attributes of pathogenic *Brachyspira* species likely consist of a set of "lifestyle" virulence factors involved in initial colonization and fitness for survival and proliferation in the microenvironment adjacent to the mucosa of the large intestine, and one or more "essential" virulence factors that are required for lesion production and/or disease. To some extent the lifestyle factors, including such things as ability to survive in an anaerobic environment, use available substrates, be motile and undergo chemotaxis are likely to be shared by commensal and pathogenic *Brachyspira* species, since all *Brachyspira* species are able to colonize the large intestine. Subtle differences in such lifestyle factors presumably cause differences in behavior—for example some *Brachyspira* species show a limited host range (e.g., *B. aalborgi* is largely restricted to humans), whilst others have a much broader host range (e.g., *B. pilosicoli* colonizes many species of birds and animals).

To date only *B. hyodysenteriae* and to a lesser extent *B. pilosicoli* have been studied in relation to their potential virulence factors (in both cases, mainly in the context of swine diseases). The host range and virulence trait determination of *B. hyodysenteriae* remains poorly understood. For example *B. hyodysenteriae* strains isolated from rheas caused severe necrotizing typhlitis and high mortality rates in rheas but were apathogenic and failed to produce significant intestinal colonization in swine (140, 154). Preliminary studies on *B. hyodysenteriae* isolates from feral mallards in Sweden suggest that these are not pathogenic in mallards or in experimentally inoculated swine (74). Similarly, some strains of *B. hyodysenteriae* recovered from swine are avirulent in experimentally inoculated swine (2, 56).

In order to colonize the large intestine *Brachyspira* species cells must penetrate and move through the mucus overlying the mucosa. All *Brachyspira* species cells are motile, but they vary in their attraction to colonic mucin. For example virulent but not avirulent strains of *B. hyodysenteriae* are attracted to mucin (104), whilst the chemotactic response of *B. pilosicoli* to mucin can be modulated by certain substrates in the growth medium (179). Some avirulent strains of *B. hyodysenteriae* lack a homologue of the *mglB* gene, encoding a glucose-galactose lipoprotein, which is believed to be a chemoreceptor in glucose and galactose chemotaxis (176). This finding supports the view that chemotaxis is involved in the expression of virulence in *B. hyodysenteriae*. The role of motility in colonization also has been confirmed by experiments in which *B. hyodysenteriae* strains with disruptions introduced to their flagella genes (*flaA* and *flaB*) had reduced motility and ability to colonize (82, 129). Similar experiments have not been conducted with the other pathogenic *Brachyspira* species, but it can be assumed that motility is important for their colonization.

Another likely "lifestyle" virulence factor of *Brachyspira* species is their NADH oxidase activity, which is believed to enhance their ability to colonize the colonic mucosa by protecting them from oxygen toxicity. Consistent with this, *B. hyodysenteriae* strains with an inactivated *nox* gene showed a reduced ability both to colonize swine and cause disease (139).

Potential "essential" virulence determinants in *B. hyodysenteriae* include this spirochete's strong hemolytic activity (56). Early studies suggested that *B. hyodysenteriae* hemolysins had molecular weights of 19 kDa, 68 kDa or 74 kDa (160). The hemolysin(s) were oxygen-stable and resembled streptolysin S, a carrier-dependent toxin. Purified hemolysin was cytotoxic to a number of tissue culture cell lines and to primary pig cells (83), and damaged epithelial cells in porcine ligated intestinal loops (96) and in the murine cecum (69). Three genes (*tlyA, tlyB* and *tlyC*) encoding putative hemolysins of *B. hyodysenteriae* were originally described, based on their ability to induce a hemolytic phenotype in *Escherichia coli* (161). It now appears that the *tly* genes may be regulatory elements, rather than encoding hemolysins themselves, but nevertheless inactivation of *tlyA* reduced both the hemolytic activity and the virulence of *B. hyodysenteriae* (70). More recently, a distinct gene (*hlyA*) has been described encoding an 8.93 kDa polypeptide of *B. hyodysenteriae* with hemolytic activity (66). *B. pilosicoli* also has the *hylA* gene, but the spirochete is only weakly hemolytic (180). This difference in phenotype between the two *Brachyspira* species may be related to differences in transcription or translation of the gene, or to a different pattern of attachment of lipid moieties to the hemolysin in *B. pilosicoli* (180). The genetic basis of the strong hemolysis of certain non-*B. hyodysenteriae* isolates from ducks and game birds has not been investigated (73, 74).

The lipooligosaccharides (LOS) in the cell envelope of *Brachyspira* species have some of the same biological properties as lipopolysaccharides from other gram-negative bacteria. LOS extracted from *B. hyodysenteriae* by phenol/water was toxic for mouse peritoneal macrophages, increased uptake of red blood cells by murine peritoneal cells via Fc and C3 receptors, acted as a mitogen for murine splenocytes, and generated chemotactic factors in fresh swine serum (114). Endotoxin extracted from *B. hyodysenteriae* by butanol/water had more biological activity than LOS extracted by phenol/water, and it induced interleukin-1 and tumor necrosis factor from murine peritoneal cells and augmented natural killer activity (45). Nevertheless, the biological activities of LOS and endoxin from *B. hyodysenteriae* and *B. innocens* are similar, and therefore may not account for the different pathogenic potential of the two species (44, 111). *In vivo* studies in mice and pigs have shown that *B. hyodysenteriae* endotoxin also induces production of pro-inflammatory cytokines such as interleukin-6 (113). Studies in mice support the view that *B. hyodysenteriae* LOS has a potential role in virulence since experimentally infected C3H/HeJ mice (hyporesponsive to LOS) showed no colonic lesions whilst C3H/HeB mice (normal responders) developed lesions (112, 114). The LOS of other pathogenic *Brachyspira* species has not been studied, other than to show that it is antigenically heterogenous amongst *B. pilosicoli* strains (93).

B. pilosicoli lacks the attachment and invasion determinants encoded by the *inv, ail* and *yadA* genes of *Yersinia enterocolitica,* the *eae* gene from enteropathogenic *Escherichia coli*, and a viru-

lence plasmid of *Shigella flexneri* (61). Although the attachment of *B. pilosicoli* to epithelial cells has been confirmed using intestinal epithelial cell lines *in vitro*, to date putative adhesins or host cell receptors have not been identified (107). Three different protease activities, including a subtilisin-like serine protease similar to that of other gram-negative bacteria have been found in the membrane of *B. pilosicoli*, but their potential role in disease causation is uncertain (25, 105).

In humans infected with *B. pilosicoli* or *B. aalborgi*, it has been suggested that the end-on attachment of a dense mat of these spirochetes to individual enterocytes can cause ablation of microvilli, with diarrhea resulting from a physical impedance to water and electrolyte absorption by the massive colonization (128). A similar situation could occur in chickens heavily colonized by *B. pilosicoli*.

Generally, the identification of virulence determinants in *Brachyspira* species has been hampered by a lack of genomic information for these spirochetes. Furthermore, experiments involving gene inactivation to analyse specific gene function have only been conducted with *B. hyodysenteriae*.

Pathobiology and Epidemiology

Host Range

Spirochetes have been recorded colonizing the ceca and recta of a variety of avian species, including chickens (63, 155, 164), common rheas (15, 16, 130), grouse (39), pheasants (99, 177), partridges (73), turkeys (131), geese (110), and captive or free-living wild birds, especially acquatic birds of the orders Anseriformes and Ciconiiformes (73, 74, 117, 148). Intestinal spirochetes have been observed in the ceca of young ostriches (*Struthio camelus*) with diarrhea (97). They have not been recorded in emus (*Dromaius novaehollandiae*), although asymptomatic emus with antibodies reacting with intestinal spirochetes have been found (154).

Incidence and Distribution

Cases of AIS in poultry have been reported in Europe, North America, and Australia; they almost certainly occur elsewhere but go unrecorded. Besides caged or housed flocks, outdoor free-range flocks also possibly are even more commonly affected (19, 175). As of 2006, necrotizing typhlitis in rheas has only been reported in the US (15, 16, 60).

There have been relatively few detailed epidemiologic surveys of AIS, and in these the incidence of colonization and disease has varied with the avian species examined and the methods used for demonstrating spirochetes. The incidence of AIS in poultry in the US is unknown, but surveys in Europe and Australia have shown the condition to be remarkably common in flocks of laying hens and broiler breeder hens. In a survey conducted in the 1980s in chicken flocks mainly from the Netherlands, using a direct fluorescent antibody test (FAT) on feces, 27.6% of flocks with intestinal disorders were positive for intestinal spirochetes whilst only 4.4% of flocks without enteric signs were positive (32). A later study in Western Australia used selective culture on feces collected from 37 randomly selected laying hen flocks and 30 broiler breeder flocks (100). Overall, 53% of the breeder hen flocks and 35% of the layer hen flocks yielded samples containing intestinal spirochetes. Moreover, 64% of the flocks with diarrhea or poor production were colonized, compared to only 24% of flocks with normal feces. Within-flock prevalence varied from 10% to 95% of the samples that were tested. More recent surveys also used selective culture, but included polymerase chain reaction (PCR) assays to identify the spirochete species present. Using these modifications, even higher overall prevalence rates were found in the eastern states of Australia, where spirochetes were recovered from 43% of 28 randomly selected broiler breeder farms and from 68% of 22 laying hen farms (142). Within these farms, most of which contained multi-aged flocks, infection was detected in 26% of 112 broiler breeder flocks (each in individual houses) and in 54% of 68 laying hen flocks. Within-flock prevalence varied from 10 to 100% of samples examined, with a mean of 47%. In this study there was a highly significant correlation between colonization and wet litter, with infected flocks on average having 14% greater fecal water content than flocks that were not colonized. Spirochetes were not detected in 45 broiler flocks on 19 farms that were surveyed. In a study in Northern Italy, 21 (72.4%) of 29 laying hen flocks were infected with intestinal spirochetes (8). In this case colonization was significantly associated with reduced egg production, but not with increased fecal water content. In the Australian and Italian studies, colonization rates were significantly higher in flocks >40 weeks of age than in younger flocks.

In surveys of various bird species held in zoological collections in the US (148) and Australia (117), colonization with intestinal spirochetes was commonly detected only in waterfowl of the order Anseriformes.

Prevalence of Pathogenic Species

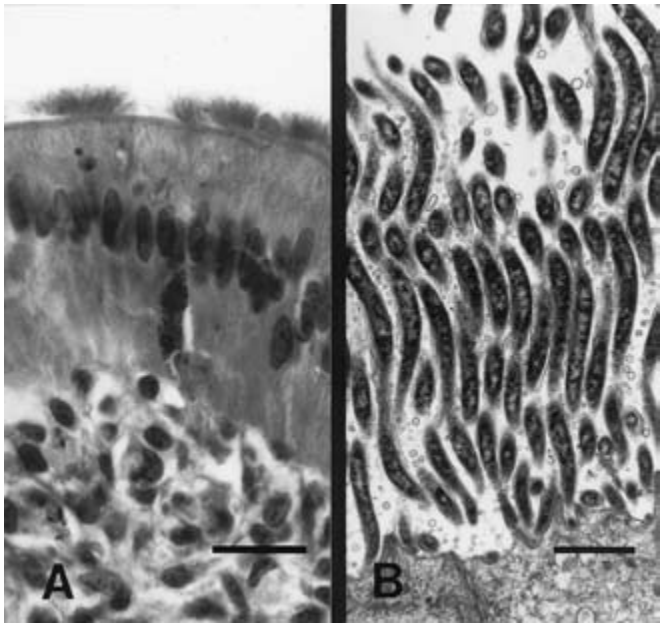
In the preceding surveys around 70% of laying flocks and 50% of breeding flocks contained birds colonized by intestinal spirochetes. Isolates from about 67% of these colonized flocks typically have belonged to pathogenic species, of which *B. intermedia* accounts for around 67% of the isolates and *B. pilosicoli* for the remainder (8, 142, 146). Individual flocks and hens may be colonized by both these pathogenic species (142, 146, 125). *B. alvinipulli* has not been detected in these surveys, and to date (2006) has only been reported in two flocks of laying hens in the US (155) and in two flocks of geese in Hungary (110). A closely related spirochete has been identified in a Swedish dog (80). Infection of rheas with *B. hyodysenteriae* is widespread in the US (17), and has been recorded in some feral and farmed mallards in Sweden (73, 74). It is suspected that infections with *B. hyodysenteriae* occasionally occur in chicken flocks, but this has not been documented.

Strains Present

In one study MLEE was used to examine multiple *Brachyspira* isolates from 4 chicken farms (146). On one farm 16 *B. murdochii* isolates were located in 14 different electrophoretic types (ETs), whilst 5 isolates of *B. pilosicoli* belonged to the same ET. On the second farm, 5 of 6 *B. pilosicoli* isolates belonged to the

same ET, and the sixth was distinct, whilst 2 *B. intermedia* isolates were different from each other. On the third farm, 3 isolates of *B. intermedia* all belonged to the same ET. On the fourth farm, the 4 *B. intermedia* isolates all belonged to different but related ETs. Hence some infected farms may have a dominant strain of a species present, but other strains of the same or other *Brachyspira* species also may be present. This heterogeneity was also found in a study on a Western Australian laying hen farm, where 20 *B. intermedia* isolates examined using pulsed field gel electrophoresis (PFGE) were divided into 4 different PFGE types (125). The existence of strain heterogeneity amongst isolates from a farm is important, as different strains may have different biologic properties that may affect the clinical outcome, including virulence traits and antimicrobial susceptibilities.

Such different strains on a farm may have been independently introduced or may have arisen from "microevolution" of original strains that were present (6). Some of the pathogenic *Brachyspira* species have been shown to have recombinant population structures and to undergo extensive genetic rearrangements and sequence drift that generates genetic diversity (173, 180). Novel genetic information also may be acquired from other species/strains through the activity of a prophage-like gene transfer agent observed in different *Brachyspira* species (67, 141).



23.13. Tight association of *Brachyspira pilosicoli* with the luminal surface of cecal epithelium (A). The orientation is at right angles to the epithelial cells (B).

Anatomic Location

Intestinal spirochetes colonize the ceca and rectum, but not the small intestine. Spirochetes primarily are found in crypt lumina and to a lesser extent in the cecal contents adjacent to enterocytes. *B. pilosicoli* cells may be found in groups attached by one cell end to the surface epithelium (Fig. 23.13), although they can colonize the ceca without attachment being seen. Spirochetemia with *B. pilosicoli* has been reported as a rare occurrence in humans but not in other animal or avian species. It is not known whether *B. pilosicoli* spirochetemia occurs in birds and goes unrecognized because of difficulties in isolating the spirochete.

Spirochete Persistence in the Ceca

Spirochetes can persistently colonize ceca (26, 33, 36). In one study spirochetes were detected in cecal droppings from the time of experimental infection at 14 weeks of age until the termination of the experiment 23 weeks later (37); in another experiment the same spirochete strain 1380 (later identified as *B. intermedia* (101)) was still present in the feces of experimentally infected laying hens after 9 months (33).

Transmission, Carriers, and Vectors

Intestinal spirochetes are transmitted between birds by the fecal-oral route, either directly or indirectly. Aerosol transmission of feces between birds that are held in close proximity in confined conditions is likely to occur. Transmission of spirochetes between flocks of birds in different houses on a farm is most likely to occur through the movement of personnel who have clothing or boots contaminated by chicken feces. Wild birds and animals such as rats and mice also potentially could introduce and/or spread infection. Insects such as flies, or species such as dogs or feral animals, might serve as mechanical carriers. A major potential source of transmission is through the water supply. Wild ducks have been shown to shed strains of *B. pilosicoli, B. hyodysenteriae* and *B. intermedia* in their feces (73, 74, 117), and these may survive in effluent ponds or dams supplying drinking water (117).

Incubation Period

The incubation period of AIS is variable. Both the dose of the organism and secondary environmental factors can profoundly influence the incubation time. Disease signs can occur in chickens as early as 5 days after experimental inoculation (157), although often it may take several weeks for significant levels of colonization and clinical signs to occur (54, 55).

Influence of Age on Spirochete Colonization

Experimentally the pathogenicity of avian intestinal spirochetes is greatest when they are given to 1-day-old birds via crop gavage (152, 157), although natural infection of such young birds is not seen. The pathogenicity of *B. hyodysenteriae* in rheas is greater for birds < 5 months old than in adults (15). In commercial laying hens it is unusual to detect colonization before 15 weeks of age, and more colonized hens are found as the flocks become older (142). This age-related distribution likely reflects increasing levels of exposure rather than differences in age susceptibility.

Influence of Diet and Microbiota on Spirochete Colonization

For *Brachyspira* species to colonize they must reach the large intestine, then establish and interact successfully within the local

microenvironment. It is assumed that the spirochetes survive passage through the upper intestinal tract inside boluses of food or feces. Studies in swine have shown that once in the large intestine the spirochetes interact with various anaerobic bacterial species forming part of the normal microbiota of the cecum and colon, including species such as *Clostridium perfringens*. These species act synergistically with *B. hyodysenteriae* to facilitate spirochete colonization and augment inflammation and lesion production (64, 79, 178).

Dietary influences on colonization with *B. intermedia* have been shown in experimentally infected laying hens. In particular, diets based on wheat seem to promote colonization with *B. intermedia* compared to diets based on barley or barley and sorghum (123). Furthermore different wheat varieties have been shown to vary in their influence on promoting colonization by *B. intermedia* (124). In one study of laying hens fed wheat-based diets the addition of exogenous enzymes designed to hydrolyse the non-starch polysaccharides in the wheat reduced *B. intermedia* colonization following experimental infection (55). In the same study, and in a subsequent study, addition of zinc bacitracin (ZnB) to the diet reduced colonization with *B. intermedia* (54). In contrast, dietary ZnB enhanced colonization with *B. pilosicoli* (71, 144). As ZnB mainly acts on Gram-positive bacteria rather than on the spirochetes themselves, these conflicting results indicate that there are likely to be complex positive and negative interactions between different components of the cecal microbiota and different *Brachyspira* species in chickens.

Taken together, these studies suggest that different clinical outcomes may occur in infected hens depending on their diet, intestinal microenvironment and microbiota, as well as the particular *Brachyspira* species involved in the colonization. These findings may help explain some of the heterogeneity in clinical signs and pathology seen in different commercial flocks with AIS.

Clinical Signs and Pathology

Information about clinical signs and pathology of AIS is limited, and available data have come from three main sources. The first is from experimental infection of 1-day-old chicks. These data provide a guide to the pathogenic potential of certain isolates, but the results must be viewed with caution as the associated colonization and disease may not be representative of natural infections in adult birds. The second source comes from experimental infection of adult chickens using defined isolates. This system is more representative of natural disease, but also has limitations. The experimental hens are usually individually caged, appropriately fed and relatively free of stress, and these conditions do not reflect the situation in many commercial caged flocks where AIS occurs. Disease is often quite mild or absent under these experimental conditions; for example, only slight reductions in egg production and/or increased fecal water content have been observed in the absence of obvious histologic changes in the ceca of some experimentally colonized birds (51, 144). Furthermore as these experiments are time consuming, testing tends to be done with only one or a few spirochete strains under a restricted set of standard dietary and other conditions. The birds are not co-infected with other species or strains of spirochetes, or with other enteric pathogens, as may occur in commercial flocks. The third source of information comes from observations of natural cases of AIS. These are important data of direct industry relevance in terms of observing changes in production, but are limited by the fact that often there may be co-infections that are unrecognized, or which make the attribution of production losses or pathology associated with AIS difficult to allocate. An example of this was a report of histomoniasis with concurrent AIS in a free-range flock where there were mortalities, cecal pathology and loss of egg production (38). Another problem with some of the earlier descriptions of AIS in the field is that the species of spirochete involved in causing the condition were not known (26, 46).

Besides the *Brachyspira* species and perhaps even strain involved in the colonization, the initiation and severity of clinical disease are influenced by the host species, husbandry, nutrition, environment, and genetics (85). Some specific predisposing factors for AIS that have been observed in the field include molting, recent onset of egg production, poor or inappropriate feed quality, floor housing, and light-laying breeds of hens (20, 46, 85, 155, 164). Crowding of birds induces stress and increases the opportunity for spirochete transmission between individuals held in close proximity.

Naturally occurring or experimental colonization of birds with intestinal spirochetes broadly may result in: 1) subclinical colonization, 2) mild to moderate clinical disease, or 3) severe clinical disease.

Subclinical Colonization

Colonization by spirochetes without disease has been reported in chickens (62, 100, 142), most usually associated with apparently commensal *Brachyspira* species such as *B. innocens, B. murdochii* and/or "*B. pulli*" (101). In wild birds, especially waterfowl, most spirochetes are not associated with enteric disease, and are considered to be commensals forming part of the normal microbiota. However, inoculation of 1-day-old chicks with some unidentified but apparently apathogenic wild bird isolates has resulted in mild transient diarrhea and yellowish green, frothy cecal contents (147, 156). From an epidemiologic perspective it is also important to note that wild birds may carry pathogenic species without showing obvious clinical signs (73, 74, 117).

Mild to Moderate Clinical Disease

The "mild to moderate" disease spectrum is seen particularly in association with strains of *B. intermedia, B. pilosicoli* and *B. alvinipulli,* mainly in laying hens and broiler breeder hens. These infections tend to be associated with diarrhea and/or reduced egg production, but cecal changes are mild or inapparent.

Infections with B. intermedia. In early studies *B. intermedia* strain 1380 was used to experimentally infect broiler chicks (34), laying hens (36) and 14-week-old broiler hens and cocks, where the eggs were collected and hatched (37). Infected chicks showed variable reductions in growth rate, wet droppings with increased fat content, and increased serum content of protein, lipid, carotenoids and biliruben (34). Laying hens showed increased fecal fat content (36), developed slimy, wet, frothy feces (33), or had wet droppings

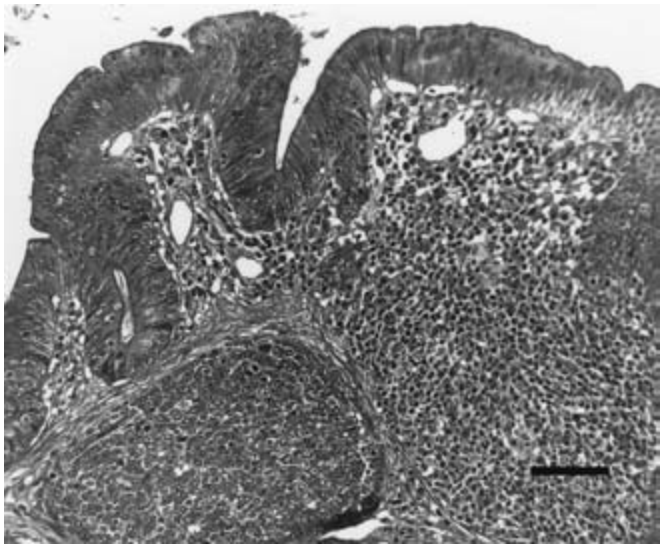
and produced significantly fewer eggs (37). Spirochetes were found penetrating the cecal mucosal lining between undamaged columnar cells, in "gap-like" lesions running through the epithelium, and accumulated just under the epithelium. There was some erosion of the superficial mucosa but no clear signs of inflammation in the connective or lymphatic tissues. The eggs from infected hens were significantly lighter, had paler yolks and had a lower carotenoid content. Broiler chicks hatched from eggs from the infected hens had pale, mucoid and wet feces, and the chicks were significantly lighter than control chicks at 2 and 3 weeks of age. They tended to develop rickets and had low blood plasma concentrations of carotenoids and alkaline phosphatase activity. They were not themselves colonized by spirochetes.

Experimental infection of laying hens with *B. intermedia* strain HB60 caused reduced growth rates, increased fecal water content, and decreased egg production and egg weight, but did not induce any characteristic pathological changes in the ceca (51, 56, 124).

Infections with B. pilosicoli. Newly hatched broiler chicks have been infected with human, porcine and/or canine isolates of *B. pilosicoli* (34, 106, 167, 168). Clinical signs either were not observed (34) or the chicks developed watery diarrhea (167, 168), sometimes with a depressed growth rate (168). Gross cecal lesions were not seen, but there were variable histologic changes included the characteristic presence of a dense mat of spirochetes attached by one cell end to cecal enterocytes (106, 167, 168), sometimes with a diffuse thickening of the cecal epithelial brush border (106). There was variable crypt elongation, crypt lumina were dilated and there was mild focal infiltration of the lamina propria with heterophils. Sometimes spirochetes were found between enterocytes or producing gap-like lesions; subepithelial accumulation of spirochetes and focal erosion without an inflammatory reaction also were recorded (34). Vacuolation and protein deposition were observed in the apical cytoplasm of some luminal enterocytes. Sometimes microvilli were obscured, damaged or obliterated by large numbers of attached spirochetes, and there was disruption to the terminal web microfilaments. Individual spirochetes invaginated into the cellular membrane and indented into the terminal web cytoplasm, but did not penetrate it.

Experimental infection of broiler breeder hens with avian *B. pilosicoli* strain CPSp1 resulted in a transient increase in fecal water content, fecal staining of eggshells and/or a significant reduction in egg production (144, 145). The ceca of infected birds were gassy and the contents were frothy, fluid and pale, but no gross or histologic lesions were observed. Spirochetes were isolated but they were not found attached to the cecal epithelium. Infection of laying hens with strain CPSp1 resulted in no disease signs (71), whilst infection with a human isolate of *B. pilosicoli* resulted in a persistent and significant increase in fecal water content (72). Again neither attachment of spirochetes nor gross pathologic changes were observed.

Natural infection of two layer flocks with *B. pilosicoli* was associated with a 5% reduction in egg production, diarrhea in up to 25% of chickens, wet droppings, feces smeared on feathers around the vent ("pasty vents"), lethargy and depression (164).

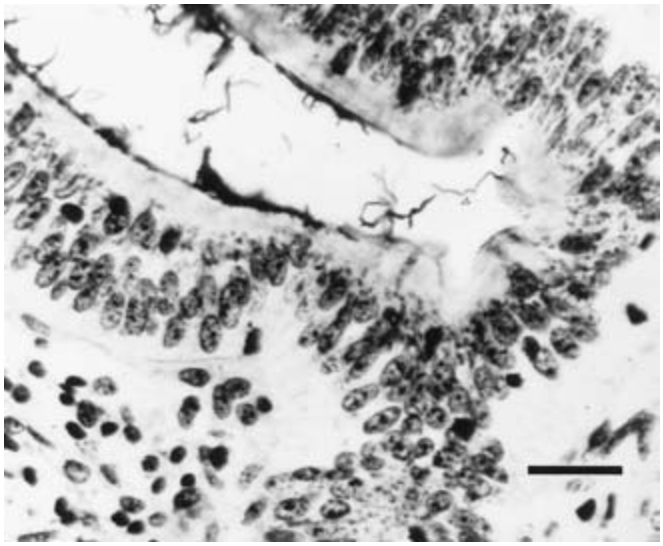


23.14. Mild lymphocytic typhlitis and mild epithelial hyperplasia in a chicken colonized with *Brachyspira alvinipulli*. Bar = 50 µm. (157)

The apical surfaces of cecal enterocytes were covered by a dense layer of spirochetes aligned parallel to each other and perpendicular to the mucosal surface (Fig. 23.13, Table 23.4). Four turkey flocks with increased mortalities were infected with *B. pilosicoli* (131). Large numbers of spirochetes were attached along the surface epithelium of the ceca and extended into the middle of crypts. Focal mucosal erosions occurred in some ceca, with spirochetes attached directly to the exposed basement membrane or invading the lamina propria. This was accompanied by an increase in the number of subepithelial mononuclear inflammatory cells.

Infections with B. alvinipulli. Experimental infection of 1-day-old chicks and 14-month-old hens with *B. alvinipulli* strain 91–1207/C1 resulted in yellow, golden or orange cecal droppings (157). The ceca were dilated and contained pale-green to yellow fluid to frothy contents. Infected birds had moderately severe lymphoplastic typhlitis and proctitis with lymphocyte and/or heterophil exocytosis, mild cecal villous epithelial cell hyperplasia, edema in the lamina propria of villous tips, and submucosal lymphocytic follicles (Fig. 23.14). Some chicks had mildly dilated cecal crypts. Mats of spirochetes were present over the villous surface and in the crypts, with spirochete cells oriented randomly on the cecal epithelial luminal surface or in the crypt lumina. Spirochetes rarely invaded between and below the cecal epithelial cells (Fig. 23.15).

B. alvinipulli strain 91–1207/C1 was originally identified in two flocks of laying hens where 5% of the chickens had wet feces, clinical diarrhea, pasty vents and produced dirty, fecal-stained eggshells (155). Spirochetes were present within the crypts and/or in the lumina of the ceca, and chickens with pasty vents had mild lymphocytic typhlitis.



23.15. Randomly oriented *Brachyspira alvinipulli* spirochetes on the villous surface epithelium in the cecum. Warthin-Starry silver stain. Bar = 20 µm.



23.16. *Brachyspira hyodysenteriae*. Thick necrotic pseudomembrane attached to the cecal mucosa of a juvenile common rhea with necrotizing typhlitis.

Cases of AIS in Which Spirochetes Were Not Identified. In an early study in the Netherlands, an unidentified weakly hemolytic spirochete designated "strain K1" was isolated from a hen on a laying hen farm where there was prolonged intermittent diarrhea and an early decrease in egg production (26). Strain K1 had too many periplasmic flagella to be *B. pilosicoli,* nor was it *B. intermedia* or *B. hyodysenteriae* because it was indole negative. Naturally infected birds had a mild typhlitis. There was a slight increase in numbers of goblet cells, focal "gap-like" lesions in the cecal epithelium that were filled with spirochetes, with mild degeneration and mononuclear cell infiltration beneath the gaps. Ten-week-old hens experimentally infected with mucosal homogenates or strain K1 showed a transient increase in fecal water content, and this recurred after 8–9 weeks.

A study in the UK reported retarded growth rate and delayed onset of egg production in 22-week-old pullets (46). The mucosal crypts of the ceca were distended with sloughed epithelial cells and inflammatory debris, and there was marked mononuclear leukocytic infiltration of the lamina. Spirochete cells were randomly oriented on the cecal epithelial luminal surface or in crypt lumina.

In the Netherlands observations were made on 8 broiler breeder flocks with a history of AIS caused by uncharacterized spirochetes (132). Flocks with clinical signs had decreased egg production and increased feed consumption. Three percent of eggs produced were too light for successful hatching. Commercial broiler flocks hatched from eggs layed in periods when clinical signs of AIS were present in the breeder flocks showed increased feed conversion and consumption. Weak chicks, retarded growth and poor feed digestion occurred in the broiler flocks. Antibiotic treatment of the breeder hens before the onset of lay resulted in normally-performing offspring.

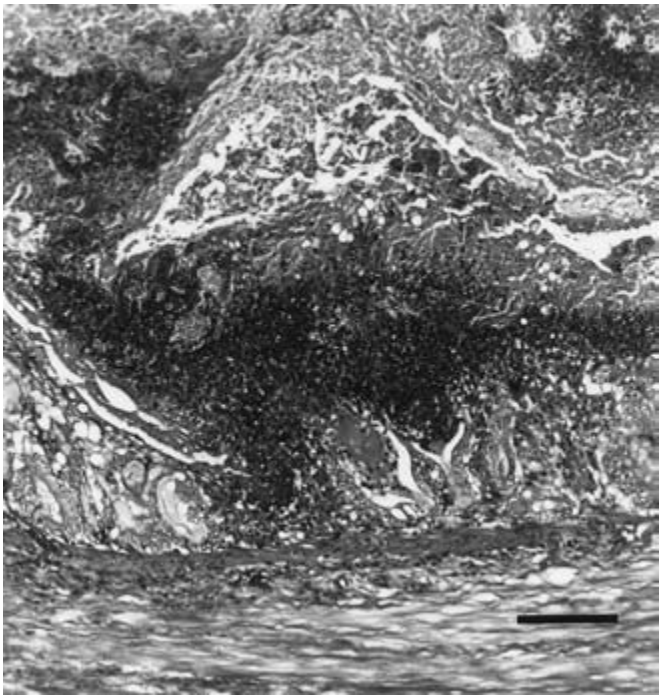
Severe Disease

Descriptions of severe disease associated with AIS have been limited to the typhlitis seen in rheas naturally infected with *B. hyodysenteriae* (16, 17, 130, 153), where mortality rates can range from 25–80%, and to a similar syndrome in geese infected with *B. alvinipulli*, where 18–28% mortality was recorded over a period of 2–3 months (110).

Most clinically affected rheas are > 6 months old (16), and most US cases occur from July through October (153). Adult birds can be affected, but these cases usually involve concurrent stress such as recent shipping. Clinically, 1–2 days prior to death, a few birds may show depression, have reduced body weights, and pass watery feces with caseous cores (152); however, rheas often die suddenly without clinical signs (130). Ceca are dilated and have thickened walls with ulcerations and lumina containing thick pseudomembranes (Fig. 23.16) (130, 152). Cecal walls have severe mucosal necrosis, crypt elongation, hyperplasia of glandular epithelial and goblet cells, and the cecal lumina contain mucus, colonies of spirochetes, bacilli, and fibrinonecrotic debris (Fig. 23.17). Experimental inoculation of 1-day-old chickens and turkeys with intestinal spirochetes from rheas produced similar but less severe lesions (77).

In the original rhea cases, a strongly ß-hemolytic spirochete was isolated and identified as *B. hyodysenteriae* (77). Inoculation of 1-day-old common rhea chicks reproduced the gross and histologic lesions within 5–9 days (152). In other cases unclassified weakly ß-hemolytic spirochetes have been isolated (130). In addition, various indigenous anaerobic bacilli can be recovered with spirochetes from cecal lesions (15). Production of AIS in rheas may require synergism between spirochetes and other anaerobic cecal microbiota.

Natural colonization of poultry with *B. hyodysenteriae* has not been reported. Experimental infection of 1-day-old chicks with porcine strains of *B. hyodysenteriae* resulted in reduced weight gain, atrophic and thickened ceca with mucus in the cecal lu-



23.17. *Brachyspira hyodysenteriae*—juvenile rhea. Thickened cecal mucosa contains dilated crypts filled with mucus. Lumen has a necrotic pseudomembrane composed of necrotic heterophils and sloughed epithelium, bacteria, and mucus. H & E. Bar = 50 µm.

mina, epithelial and goblet cell hyperplasia, and crypt elongation (3, 149, 150, 167). There was goblet cell hyperplasia, necrosis of the epithelium at tips of plicae, abundant spirochetes in crypts, and edematous lamina propria with accompanying heterophilic inflammation.

Severe disease associated with *B. alvinipulli* infection has been recorded in two goose flocks in Hungary (110). Following molting at the end of the first egg laying season, 28% of the 1,500 laying birds in flock A died during an 8-week period and 18% of the 4,500 laying birds in flock B died during a 12-week period. Affected geese had hemorrhagic to necrotic inflammation of the colon/rectum and fibrinonecrotic typhlitis accompanied by severe degeneration. Spirochetes were present in the mucous membrane of the large intestine. The kidneys were swollen, and some geese had visceral gout. The large intestine had a necrotic epithelial layer and the lamina propria contained hemorrhage as well as infiltration with lymphocytes, histiocytes and heterophilic granulocytes. Sometimes necrosis extended into the upper third of the lamina. Kidneys had degeneration of the tubular epithelial cells, focal or diffuse intertubular fibroblast cell proliferation, with atrophy of the glomeruli and tubules, and mineral deposition. Lymphohistiocytic inflammation of the liver was observed. Nine isolates were identified as *B. alvinipulli* whilst another (from flock A) was strongly betahemolytic but indole negative, and was tentatively identified as *B. hyodysenteriae.*

Immunity

Little is known about immunity to intestinal spirochetes in birds, and, as previously stated, prolonged colonizations of individual experimentally infected birds have been observed (33). Humoral antibodies to *Brachyspira* spp. preparations may or may not be produced following naturally occurring colonization. Antibodies can be detected in birds from which spirochetes cannot be isolated, and other birds may yield spirochetes on culture but be serologically negative (147, 148).

Diagnosis

Introduction

Gross pathologic and histologic examinations are rarely sufficient to allow an unequivocal diagnosis of AIS. Hence the diagnosis of AIS is usually confirmed using microbiological techniques to identify the associated spirochetes in birds with clinical, pathologic and/or production data consistent with AIS.

Visualization of Spirochetes

Visual demonstration of helical-shaped bacteria in feces or cecal droppings by dark-field or light microscopy is sufficient for presumptive identification of spirochetes. However, because spirochetes can be normal microbiota or produce subclinical colonizations, characteristic clinical signs and lesions must be present for a presumptive diagnosis of AIS.

Confirmation of bacteria as spirochetes should be through visualization of distinctive ultrastructural features, demonstration of spirochete antigens, isolation in culture, or by polymerase chain reaction (PCR) on feces. The demonstration of organisms with periplasmic flagella in ultrathin sections of cecal mucosa or in negative-stained preparations of clarified cecal contents is diagnostic for spirochetes. Demonstration of spirochete antigens by direct or indirect fluorescent antibody tests (IFAT) (26, 32, 92) or immunohistochemical (IHC) methods using polyclonal antisera is easier and more rapid than electron microscopy (42, 177). IFAT using antiserum raised against *B. hyodysenteriae* was used in the early epidemiologic surveys for AIS (26, 32). However, neither ultrastructural morphology nor identification of antigens by IFAT or IHC using polyclonal antisera will reliably distinguish between spirochete groups or species. Monoclonal antibodies (Mab) to cell envelope proteins of *B. pilosicoli* have been described (92, 159), and these could increase the specificity of IFAT for identifying this species in chickens. Similar reagents are needed for the other avian pathogenic spirochete species. Unfortunately, a Mab-based immunomagnetic separation of *B. pilosicoli* from swine feces did not improve sensitivity of detection above that achieved with standard culture followed by PCR (23). The direct use of PCR on chicken feces is discussed later.

Isolation of Causative Spirochetes

Culturing and further characterization is important to help identify the spirochete species, and to allow strain typing and determination of antimicrobial sensitivity. The level of detection of

culture is dependent on the number of organisms and type and condition of the sample. Fresh cecal droppings or cecal mucosa are optimal samples, but samples chilled at 4°C for up to 1 week are acceptable.

Identification of Causative Spirochetes

Phenotypic Properties

Isolated bacteria can be confirmed as spirochetes by their characteristic morphology and motility under dark-field or phase contrast microscopy, the presence of periplasmic flagella observed under transmission electron microscopy, and/or their reactivity in immunofluorescent microscopy using specific antisera (see above). As discussed earlier under "Etiology", the observation of hemolytic patterns on blood agar and the patterns of biochemical reactivity can allow a presumptive identification of some *Brachyspira* species (Table 23.4).

Genotypic Properties

The use of molecular methodology for spirochete identification (and detection) has greatly improved diagnostic capacity. MLEE has been extremely useful in defining the current *Brachyspira* species (94, 101, 146), but is too slow and cumbersome for routine diagnosis.

PCR assays have been used on isolated spirochetes or on the growth on primary isolation plates to identify and differentiate *Brachyspira* species (5, 7, 95, 151). Laser capture has also been used to recover *B. pilosicoli* from fixed cecal mucosa of turkeys prior to PCR amplification (131). To date the most reliable PCR assays for amplification of DNA from *B. pilosicoli* have been based on the 16S rRNA gene, whilst a recently improved PCR based on the NADH-oxidase (*nox*) gene works well for *B. intermedia* (125, 126). Both *nox* and *tly* gene PCRs are regularly used for *B. hyodysenteriae* (41, 89). No PCR assays have yet been described for *B. alvinipulli*.

A number of other schemes have been developed to detect and/or identify *Brachyspira* species without the need for culture and biochemical analysis. These involve PCR amplification of specific gene sequences, followed by restriction enzyme digestion of the products to give species-specific banding patterns after gel electrophoresis. Genes that have been used in this way for identifying different (mainly swine) *Brachyspira* species include the 16S rRNA gene (137), the 23S rRNA gene (9, 162), and the *nox* gene (127, 163).

Another recent adjunct to diagnosis has been the development of a fluorescent *in situ* hybridization (FISH) technique. This uses fluorescent oligonucleotide probes specific for sequences present in the 16S or 23S rRNA of different *Brachyspira* species to visualize spirochetes associated with the mucosa in formalin-fixed tissues (11, 78). Recently this technique has been further modified so that visualized spirochetes are isolated by laser capture microdissection, subjected to direct 16S rRNA gene PCR and the subsequent DNA sequence analysed (84). The advantage of these techniques is that they provide simultaneous identification and localization of the spirochetes associated with the intestinal mucosa. They should prove particularly useful in investigating aspects of the pathogenesis of AIS.

Strain Typing

Typing of individual *Brachyspira* species strains can provide important epidemiologic information to help devise control measures. Early studies used MLEE to differentiate intestinal spirochete isolates into species and strains (94, 101, 136), but this technique is not particularly good for discriminating all strains, and is time-consuming. Pulsed field gel electrophoresis now is the most commonly used strain typing technique for *B. pilosicoli, B. intermedia* and *B. hyodysenteriae*, and gives better strain discrimination than MLEE (4, 6, 125, 151, 173).

PCR on Feces

Recently, a two-step nested duplex PCR has been described for detection of *B. intermedia* and *B. pilosicoli* in DNA extracted directly from washed chicken feces (126). The first round of PCR amplifies genus-specific portions of the 16S rRNA and *nox* genes, whilst the second round uses a nested *B. pilosicoli*-specific 16S rRNA gene PCR and a *B. intermedia* specific *nox* PCR. Washing removes potential PCR inhibitors, and a two-step amplification procedure compensates for any loss of sensitivity associated with this washing step. This assay is rapid and should enhance diagnostic capacity for AIS. It could be further improved, and modified to include a *B. alvinipiulli*-specific PCR in the second round of amplifications.

Serology

Several serologic tests have been developed and used to determine exposure of swine to *B. hyodysenteriae*. Such tests have not used species-specific antigens and often have had low specificity and/or sensitivity (88). They include enzyme-linked immunosorbent assays, plate and microagglutination tests, agar gel diffusion, passive hemolysis assays, and indirect fluorescent antibody tests (56). An agar gel diffusion test has been used to identify evidence of intestinal spirochete colonizations of birds (148). The test does not, however, distinguish between different spirochete species and is relatively insensitive.

Differential Diagnosis

Spirochetes identified in fecal specimens from poultry should be distinguished from other helical bacteria including *Campylobacter, Arcobacter, Helicobacter,* and *Spirillum*. Many helical bacteria can be apathogenic indigenous flora of the gastrointestinal tract of birds. Wet droppings have a number of known causes, and these may interact in a multifactorial way to result in severe problems. In cases of chronic diarrhea or pasty vents, nutritional problems such as excess dietary salt, fats, or raw soybean meal should be investigated. Increased urinary output due to excessive drinking, kidney damage, and incorrect amounts of calcium and electrolytes in the diet can result in wet litter, as can water spillage. Other infectious causes of chronic diarrhea include enteric salmonellosis, colibacillosis, coccidiosis and histomoniasis.

In rheas and geese, intestinal spirochetes causing a necrotizing typhlitis must be distinguished from other potential causes including *Salmonella*, especially group B serotypes, *Clostridium difficile, C. perfringens, C. sordelli*, and *Histomonas meleagridis* (28, 102, 133). Furthermore, cecal lesions of eastern equine en-

cephalitis virus (EEEV) infection could be confused with severe AIS, but EEEV also produces copious small intestinal hemorrhages and necrosis and widespread petechiation and necrosis in visceral organs (174).

Intervention Strategies

Biosecurity

In farms that do not have AIS, strict biosecurity measures should be in place to prevent entry. There should be good physical containment (security fencing) around the farm, and bird-proof netting around openings in the houses. Entry of personnel should be restricted, preferably with shower-in and shower-out facilties. Replacement hens should only be obtained from sources known to be free of AIS. The food and water supply should be free of contamination. In particular, the water supply should be protected from fecal contamination from feral waterfowl.

Management Procedures in Flocks with AIS

Farms with AIS should practice the same precautions as outlined previously, but in addition they should decrease contact with potentially infectious feces by raising hens off floors, frequently changing litter and removing manure, implementing good rodent and insect control programs, minimizing dietary and molting stress, and providing high-quality feed ingredients whilst avoiding ingredients that enhance spirochete colonization (e.g., wheat). To prevent transmission between flocks on a site, and particularly from older to younger flocks, clean coveralls and boots, and disinfectant boot-dips should be provided at the entry into each house.

In the case of rheas, it is best to avoid raising them on swine farms. Visiting other rhea farms should be discouraged. Proper cleaning and disinfection of clothing, shoes, and equipment should be done before returning to the home flock following visits to rhea shows, rhea farms, or swine operations. All introductions of new rhea stocks should follow a minimum 60 days of quarantine and take place after 2–3 negative cloacal cultures for *B. hyodysenteriae.* Birds should be segregated into age groups, and strict biosecurity measures should be implemented to minimize potential transmission of *B. hyodysenteriae* from asymptomatic adolescent or adult birds to susceptible rhea chicks.

Vaccination

No vaccines are available to prevent AIS. Commercial bacterins and vaccines have been used to help control swine dysentery, but these have either provided inconsistent protection (53, 57) or even had deleterious consequences (116). Experimental vaccines using live attenuated strains of *B. hyodysenteriae* (70, 129, 139), recombinant proteins (90) or DNA (27) have all been developed, but have not yet reached commercial production.

Antimicrobial Treatment

Introduction

The authors are not aware of any chemotherapeutic compound that has been approved and registered for the treatment or prevention of AIS. Nevertheless, compounds used to treat or prevent swine dysentery and/or porcine colonic spirochetosis should have similar efficacy for treatment of AIS (48, 56).

In Vitro *Testing of Antimicrobials*

There are only two publications on *in vitro* antimicrobial susceptibility testing of intestinal spirochetes from birds. The first US study examined two isolates of *B. pilosicoli* and two of *B. alvinipulli* from chickens, and three isolates of *B. hyodysenteriae* and one of uncertain identity from rheas (165). The isolates were tested in agar dilution for their susceptible to 11 antimicrobial agents that are commonly available to commercial poultry producers or have been successfully used to treat *Brachyspira* species infections in swine. All 8 isolates were susceptible to tiamulin, lincomycin and carbadox, resistant to streptomycin, and gave strain dependant results for chlortetracycline, oxytetracycline, tylosin, bacitracin, erythromycin, neomycin, and penicillin.

A second larger study investigated susceptibilities of predominantly Australian isolates *B. intermedia* (n=25) and *B. pilosicoli* (n=17) from chickens (59). These were tested in agar dilution against four concentrations each of the antimicrobials tiamulin, lincomycin, tylosin, metronidazole, tetracycline and ampicillin. Isolates of both species generally were susceptible to tiamulin, lincomycin, metronidazole and tetracycline. The *B. intermedia* isolates tended to be less susceptible to tiamulin and more susceptible to lincomycin, tylosin and ampicillin than the *B. pilosicoli* isolates. Although not classified as resistant, four isolates of *B. intermedia* had an elevated MIC range for tiamulin (1–4 mg/L), 11 isolates of *B. intermedia* and five of *B. pilosicoli* had an elevated MIC range for lincomycin (10–50 mg/L), one isolate of *B. pilosicoli* had an elevated MIC range for tetracycline (10–20 mg/L), and one isolate of *B. intermedia* and five of *B. pilosicoli* had an elevated MIC range for ampicillin (10–50 mg/L). A clear lack of susceptibility to tylosin (MIC >4 mg/L) was seen in 11 isolates each of *B. intermedia* and *B. pilosicoli*, and to ampicillin (MIC >32 mg/L) in two isolates of *B. pilosicoli*.

These *in vitro* data suggest that drugs such as tiamulin, lincomycin and metronidazole should prove useful in the treatment of AIS, regardless of the *Brachyspira* species involved. Nevertheless it is recommended that *in vitro* susceptibility testing be undertaken on several representative isolates before antimicrobial therapy is contemplated. To avoid possible toxicity, tiamulin should not be used in combination with ionophores (e.g., monensin, salinomycin and narasin).

In Vivo *Treatment with Antimicrobials*

Treatment of laying hens with some antimicrobials is problematic because of the withdrawal times needed to avoid residues being present in the eggs. In addition, drugs such as the nitroimidazoles are not available for use in food producing animals in many legislative areas, most notable in the European Union and the US, because they are genotoxic (43). Finally, as previously stated, no antimicrobials have been specifically registered for the treatment of AIS in hens. Nevertheless, several reports are available on the outcome of antimicrobial treatment of flocks with AIS, as well as of treatment of individual experimentally infected hens.

In a study on a UK laying hen unit with AIS, treatment of immature hens with 125 ppm dimetridazole in-feed for 10 days resulted in improved condition and egg production, and spirochetes were not isolated at postmortem (46).

In the Netherlands, in-water treatment of infected broiler breeder flocks with 120 ppm Ridzol S™ (a 5-nitroimidazole) for 6 days resulted in a sometimes temporary increase in egg production (132). Lasting effects required early treatment, whilst late treatment did not improve production. Reinfection of birds in some flocks may have resulted from contact with infected litter or from ineffective treatment of parts of a flock. Longer intervals between medications were suggested to increase the numbers of spirochetes shed in the feces.

In an Australian study, two houses each of 8,000 40-week-old broiler breeder hens with AIS were treated with antimicrobials in the water (142). House 1 received lincospectin at 50 mg per bird per day for 7 days, whilst house 2 received tiamulin at 25mg/kg body weight for 5 days. Treatment with lincospectin resulted in slimy feces persisting for several weeks. Hens in the lincospectin-treated house remained negative for spirochetes for 3 months, after which 30% of fecal samples were spirochete positive. Three weeks after tiamulin treatment approximately 30% of fecal samples from the house again became spirochete positive, increasing to 80% after another 3 months. Both houses were then water medicated with oxytetracycline at 60 mg/kg for 4 days. This removed the low level of infection from house 1, but only reduced the prevalence from 80% to 60% in house 2. Subsequently the prevalence in house 2 built up to 70% after 4 weeks. It was assumed that reinfection occurred either from the environment of the houses or from birds that had not received adequate medication to remove the infection. Effective control may require regular treatments with courses of antimicrobials, for example given at 1–2 month intervals, together with thorough house cleaning and implementation of strict biosecurity measures to prevent spread of infection between houses.

In a recent UK study, three flocks of approximately 12,000 laying hens on a multi-age site were found infected with *B. pilosicoli* (20). In-water treatment with tiamulin at 12.5 mg/kg body weight for 3 days resulted in increased egg production and reduced mortality.

In laying hens experimentally infected with *B. intermedia,* both zinc bacitracin (ZnB) at 50 ppm in the food and 256 pp of a dietary enzyme designed to hydrolyse the non-starch polysaccharides in wheat (Avizyme® 1302) resulted in less colonization (55). In a subsequent experiment, 100 ppm ZnB inhibited colonization with *B. intermedia*, whilst hens treated with tiamulin at 25 mg/kg body weight for 5 days became spirochete negative and maintained egg production, although they later became reinfected (54). The use of ZnB is not necessarily recommended for the control of AIS, as 50 ppm in the food resulted in an increased susceptibility of laying hens to infection with *B. pilosicoli* (71). Treatment of broiler breeder hens with either tiamulin at 25 mg/kg body weight for 5 days or with lincomycin at 20 mg/kg for 5 days removed experimental infection with *B. pilosicoli* (145).

Taken together, these studies suggest that treatment with tiamulin, lincomycin/lincospectin, dimetronidazole or even chlortetracycline should assist with control of AIS in adult hens.

For rheas with severe AIS, treatment with dimetridazole (25–50 mg/kg body weight once or twice daily), lincomycin (25 mg/kg twice daily), or erythromycin (15–25 mg/kg once daily) for 5–7 days has been successful in reducing illness and deaths (60).

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Tuberculosis

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Introduction

Tuberculosis of poultry, often termed avian mycobacteriosis, avian tuberculosis, avian TB or TB, is a contagious disease caused by *Mycobacterium avium*. Avian tuberculosis is a chronic infection. Persistence in a flock, once established, induces unthriftiness, decreased egg production, and finally causes death. Although tuberculosis in commercial poultry in the United States is rarely diagnosed, tuberculosis still occurs sporadically in backyard poultry and game birds, and it remains an important problem in captive exotic birds.

Public Health Significance

The literature contains a number of instances in which it was claimed that *M. avium* was responsible for a tuberculous infection in humans. In the United States, the first case of avian tuberculosis in humans (with adequate proof) was published in 1930 (30).

With a decline in the incidence of tuberculosis due to *M. tuberculosis* in humans, increasing interest has been directed toward other mycobacteria, i.e., *M. avium* (27, 117, 131). Moreover, *M. avium* infections have been common in patients with acquired immune deficiency syndrome (AIDS) (22, 28, 58, 128). In the United States, *M. avium* serovars 1, 4, and 8 are isolated most frequently from AIDS patients, and serovars 4, 8, 9, 16, and 19 are isolated most frequently from non-AIDS patients (28). *M. avium* serovar 1 commonly is isolated from wild birds as well as AIDS patients (39, 49). Based on pulse-field gel electrophoresis, *M. avium* isolates recovered from humans and animals have some relatedness, but the human isolates are more closely related to pig isolates rather than those from birds (10, 31). *M. avium* serovar 2, the organism most frequently isolated from chickens, is rarely isolated from humans. Thus, it would appear that most human *M. avium* infections would more likely be due to human-to-human contact rather than bird-to-human contact.

History

Tuberculosis in chickens was first described in 1884 (20). Initially, Koch (60) maintained that tubercle bacilli were a single species irrespective of host. However, Rivolta and later Maffucci (67) showed that the microorganism of tuberculosis in chickens was dissimilar to that of bovine tuberculosis. Eventually, Koch (61) declared that tuberculosis of poultry was unlike tuberculosis of humans and that the disease in humans was dissimilar to that of cattle.

Cases of avian tuberculosis in domestic poultry have declined with the development of integrated commercial poultry farming. Cases in chickens and turkeys are predominantly in hobby flocks. The occurrence of avian tuberculosis in birds in zoo aviaries has become an important disease with increasing economic costs. Certain species of exotic birds have increased in value as they near extinction, thereby increasing the significance of mortalities from avian tuberculosis. Management problems concerning control of the disease are magnified, because exotic species are often maintained for years. The ability of the *M. avium* to survive in the soil and the lack of adequate procedures for cleaning and disinfecting contaminated premises have become a major obstacle to the elimination of avian tuberculosis from zoologic collections. This is made more complex by the lack of efficacious vaccines or suitable drug-treatment regimens.

Etiology

The most common cause of avian tuberculosis in chickens in the United States is *M. avium* serovars 1 and 2 (110). In Europe, *M. avium* serovar 3 has been isolated from wild birds (74, 77) and in Japan serotype 9 (*M. avium* subspecies *hominissuis*) has also been isolated from painted quail (75, 99); however, neither of these organisms has been isolated from domestic birds in the United States (91), with the exception of a single imported White-headed tree duck (*Dendrocygna viduata*) from which serotype 3 was isolated (119). The subspecies (sbsps) classification of bacteria belonging to *M. avium* in recent years has further been clarified with the advent of molecular techniques. This classification has further elucidated correlation of certain characteristics such as host predilection and pathogenicity to particular subspecies. Historically the determination of the etiological agent involved isolation, biochemical typing, and determination of polar glycolipid surface antigen type by agglutination. Newer methods of classification have allowed *M. avium* serovars 1, 2 and 3 to be reclassified as *M. avium* sbsps *avium. M. avium* sbsps *avium* may further be identified as *M. avium* IS*1245*+ and IS*901*$^{+}$ or *M. avium* IS*1245* RFLP "bird type", or combination of these (4, 24, 79, 81, 99). This has allowed the proposed delineation of *M. avium* sbsps *avium* into *M. avium* sbsps avium and *M. avium* sbsps hominissuis depending on their molecular classification (24, 79, 98, 134). *M. avium* sbsps *avium* is known to be virulent for chickens (4, 24, 71, 79, 122). For the purposes of this text, the agent of avian tuberculosis in poultry will be referred to as *M. avium.*

Growth Requirements and Colony Morphology

In contrast to *M. tuberculosis* and *M. bovis* (102), *M. avium* grows at temperatures ranging from 25–45°C, although the most favorable temperature range is 39–45°C. *M. avium* is aerobic. However, for primary isolation, growth is enhanced by an atmosphere of 5–10% carbon dioxide (113).

Special media (113) designed for culturing tubercle bacilli is desirable for isolation from field materials. Colonies are larger if the medium contains glycerin. Some subspecies of *M. avium* such as *paratuberculosis* and *sylvaticum* require mycobactin as a growth factor for initial and subsequent growth (69). On media containing whole egg or egg yolk and incubated at 37.5–40°C, small, slightly raised, discrete, grayish white colonies form in 10 days to 3 weeks. If the inoculum has abundant bacteria, colonies will be numerous and coalesce. Colonies are hemispheric and do not penetrate the medium. They gradually change from grayish white to light ocher and become darker as the age of the culture increases. In one instance, bright yellow colonies have been described (56).

Subcultures on solid media result in growth within 6–8 days and reach maximal development in 3–4 weeks. Such cultures usually appear moist and unctuous; the surface eventually becomes roughened. The colonies are creamy or sticky and are readily removable from the underlying medium. In liquid media, growth occurs at the bottom of the tube as well as at the liquid surface. Recently, three culture media were evaluated to determine the best media to use when culturing tissue and fecal samples from *M. avium* infected Japanese quail. Modified Herrold egg yolk with mycobactin, Lowenstein-Jensen, and Lowenstein-Jensen with cycloheximide, nalidixic acid and lincomycin were evaluated. Lowenstein-Jensen media (without additives) provided more positive cultures, had greater numbers of colonies on

positive tubes and had shorter incubation times than the other media (106).

A definite relationship appears to exist between the type of colony and virulence (6). *M avium* with smooth transparent (SMT) colonies were virulent for chickens; in contrast, variants with smooth-domed (SMD) or smooth-opaque (SMO) or rough colonies (RG) were avirulent regardless of source (6, 108). Colony morphology is transient and variable and other colony morphologies have been observed although with lesser frequency (86). These different colony morphologies also vary in their ability to stimulate oxygen radicals, to multiply within the cells, and their capacity to up- or down- regulate cytokines/chemokines (8, 53, 86, 96). Differences in colony morphology are mostly due to the presence in SMT or the absence in RG of glycopeptidolipids. Glycopeptidolipids seem to limit macrophage response and may be key virulence factors (8).

The most characteristic morphologic feature of *M. avium* is its acid-fastness. The organisms are bacillary in morphology; but club-like, curved, and crooked forms are also seen in some preparations. Cords are not formed. Branching infrequently occurs. Most of the bacteria have rounded ends and vary in length from 1–3 mm. Spores are not produced, and the organisms are nonmotile. Spherical or conical granules occur anywhere within the cytoplasm.

Biochemical Properties

There appears to be no significant biochemical differences between *M. avium* and *M. intracellulare*. The *M. avium* and *M. intracellulare* group (MAIG), however, do have features that separate them from other species or groups of mycobacteria (26).

MAIG does not produce niacin, does not hydrolyze Tween-80, is peroxidase-negative, produces catalase, does not have urease or arylsulfatase, and does not reduce nitrate; there are variations in these features, particularly in the results of tests for arylsulfatase. MAIG lack most amidases except for pyrazinamidase and nicotinamidase. Detailed listings of the biochemical features of MAIG and related microorganisms are available (89, 113).

Further identification of mycobacterial cultures may be performed using high-performance liquid chromatography (HPLC) for the identification of mycolic acids (21) or through the use of molecular tests such as 16S rRNA, *hsp65* and *groEL2* sequencing, as well as polymerase chain reaction (PCR) detection of IS elements (65, 125).

Disease in poultry is mostly attributed to one subspecies, namely *M. avium* sbps *avium*. However, in pet and wild birds, speciation of mycobacterium isolates is warranted as infection has been reported with several subspecies of *M. avium* and species of *Mycobacterium* including *M. tuberculosis, M. bovis, M. fortuitum,* and *M. genavense* (6, 9, 10, 14, 28, 34, 45, 46, 47, 55, 56, 57, 69, 84, 101).

Sensitivity to Antituberculosis Drugs

Generally, *M. avium* is more resistant to the commonly used antituberculosis drugs as compared to *M. tuberculosis* and *M. bovis* (26). However, synergistic effects of antimycobacterial drug combinations (i.e., ethambutol and rifampicin) on *M. avium* complex have been reported (45). This generalized increase in resistance is attributed to its lipid rich cell wall (63, 80). This is further proven by the correlation between colony phenotype and the extent of antimicrobial resistance. Transparent colonies are always more resistant than their opaque counterparts (51, 85). The ability to bind the lipoprotein Congo red further demonstrates the association of resistance in *M. avium* and its cell wall: white colonies, which do not bind lipoprotein, are more resistant than red colonies (16, 80).

In approximately 50 strains of *M. avium* from chickens and swine and 11 from humans, in egg yolk agar, most strains will grow in the presence of 10 mg, but not in 50 mg, of streptomycin/mL, in more than 10 mg of *p*-aminosalicylic acid/mL, and in more than 40 mg isoniazid/mL medium. On the same kind of medium, *M. avium* is relatively resistant to ethambutol, thionamide, viomycin, and pyrazinamide. The inhibitory concentration is variable, depending on the medium and procedure (35).

Strain Classification

M. avium belongs to the slow growing nontuberculous bacteria group and together with *M. intracellulare* form the *Mycobacterium* avium complex (MAC). MAC organisms' reservoir is the environment. All members of the MAC have been isolated from animals. Strains of *M. avium* have traditionally been identified by serologic procedures (90). A numbering scheme was developed on the basis of the production of similar polar glycolipid surface antigens for reporting MAC serotypes or serovars (133). Serological typing allows for the recognition of at least 28 serovars of *M. avium* (serotypes 1 to 6) and *M. intracellulare* serotypes 7, 12 to 20, and 22 to 28) (129). *M. avium* can be further subdivided into *M. avium* sbsps *paratuberculosis* isolated from ruminants and free ranging birds (2, 11), *M. avium* sbsps *silvaticum* isolated from wood pigeons and other exotic birds, and *M. avium* sbsps *avium* isolated from birds and other domestic animals (25, 68). While *Mycobacterium avium* sbsps *paratuberculosis* has been recovered by culture from wild birds it has only been shown to cause lesions in one crow in spite of testing over 250 birds (5, 19). In 2002 Mijs (73) and collaborators suggested the *M. avium* sbsps *avium* can be further subdivided based on phenotypic and genotypic grounds into *M. avium* sbsps *avium* for isolates originating from birds (serovars 1, 2 and 3) and *M. avium* sbsps *hominissuis* (serovars 4, 6, 8 to 11, and 21) for isolates recovered from humans and animals (1, 25, 37, 68, 107). Serovars 1 and 2 occur mainly in animals, whereas 4 to 20 are commonly found in humans (111). Some serovars of *M. avium* found in swine (serovars 4 and 8) have also been isolated from humans (132). Serovars 1 and 2 are most commonly isolated from chickens, and serovar 3 is recovered sporadically from wild birds (74, 77). Distinguishing serovars provides a means for studying origin and distribution of specific strains. This typing method is simple and can be conducted in microtiter plates (118). However, not all isolates can be reliably serotyped using this method as some are untypable (13). In the past few years it has been demonstrated that a combination of molecular techniques is by far a more accurate method of classifying *M. avium* isolates. Strains are identified based on the presence or absence of insertion elements (IS) and are further subdivided by restriction fragment length polymorphisms

(RFLP). All *M. avium* sbsps *avium* are of the genotype IS*1245*$^+$, IS*901*$^+$, IS*1311*$^+$, and have the IS*1245* RFLP unique 3 band pattern called "bird type" for those isolates from birds which correspond to serovars 1, 2 and 3. *M. avium* sbsps *sylvaticum* has the same genotype except that the IS*1245* RFLP differs in band sizes (23, 24, 79, 81, 99). The presence of IS*901*$^+$ in isolates has been associated with virulence for birds (79). Molecular techniques with more accurate strain classification allow better epidemiologic study of these organisms (24, 25, 48, 62, 79, 98, 105). With future progress of molecular tools newer and more discriminatory typing methods for this dynamic group of organisms will emerge (125).

Pathobiology and Epidemiology

Incidence and Distribution

Avian tuberculosis in chickens is caused by *M. avium* serovars 1, 2, and 3, is worldwide in distribution, but occurs most frequently in the North Temperate Zone (121). As stated previously, avian tuberculosis is diagnosed rarely in commercial poultry. It has been diagnosed in small flocks from 30–400 chickens in Canada (76), and in relatively small free-range commercial flocks of 2000 chickens in Australia (37) and in a commercial egg laying flock in Spain (38). Historical evidence has shown that the highest incidence of infection in the United States was found in flocks of the north central states—North Dakota, South Dakota, Kansas, Nebraska, Minnesota, Iowa, Missouri, Wisconsin, Illinois, Michigan, Indiana, and Ohio. Incidence of the disease in western and southern states is low. The explanation for this is not entirely obvious, although there are several possible contributing factors such as climate, flock management, and duration of infection. The necessity of keeping birds closely confined during winter provides favorable conditions for the spread of the disease.

The difficulty of tuberculin-testing all chickens in the United States, or even a majority of the flocks, makes it impossible to obtain exact data on the incidence of *M. avium* infection of chickens. Slaughter data maintained in the United States by the National Agricultural Statistics Service for the years 1995 through 2005 revealed that avian tuberculosis was the cause for the condemnation of young chickens only in 1997 and 1998 at a rate of 7.5 and 6.2 birds per 10,000,000 birds slaughtered, respectively. In mature chickens during the same period, avian tuberculosis was diagnosed during 1996, 1999, 2000, 2001, 2002, 2003, 2004, and 2005 at a rate of 2.1, 1870, 1630, 14.6, 0.59, 2.4, 18.4, and no birds per 10,000,000 slaughtered, respectively. Avian tuberculosis was diagnosed in mature turkeys only in 2003 at a rate of 0.04 per 10,000,000 turkeys (64, 83). Because visual inspection alone is used to derive these numbers, this figure may represent an under- or overestimation of the true incidence. The diagnosis at inspection was most likely based only on emaciation and the presence of granulomas (11).

From 1985 to 2001, a midwestern animal diagnostic laboratory (Fulton, unpublished data) received 6,059 avian submissions involving 15,097 birds. Only 27 (0.45%) cases involving 36 (0.24%) animals were diagnosed with tuberculosis. Of these cases, only 3 (0.05%) cases (4 animals) were chickens from hobby flocks; 2 cases were in peafowl; and 1 case each in pigeons, doves, quail, and partridges. By far, the largest group represented was exotic captive birds (parrots, toucans, budgerigars, and finches). Three different zoos had diagnoses of avian tuberculosis in such species as penguins, a crane, a duck, an ostrich, and toucans. No cases were seen in commercial chickens or turkeys.

Avian tuberculosis also occurs in some Latin American countries such as Brazil, Uruguay, Venezuela, and Argentina.

Overall, there has been significant reduction in the prevalence of avian tuberculosis due in part to the changes in poultry husbandry. Increasing emphasis has been placed on the desirability of maintaining all-pullet flocks, rather than older hens.

Historical information from the 1960s reports infection of animals by *Mycobacterium avium* in certain European countries. It is said to be rare in Finland (127), but not uncommon in Norway (33) and Denmark (2); *Mycobacterium avium* infection occurs in Germany (71, 93) and Great Britain (66). In Australia, avian tuberculosis is unknown in Queensland and West Australia but occurs in other states. In South Africa, the incidence in poultry is low (59). Infections probably occur in domestic and wild fowl in other countries, but the incidence and distribution cannot be determined because bacteriologic studies are not universally done. In Kenya, avian tuberculosis has been reported in lesser flamingoes (18). Historical prevalence of tuberculosis in animals may be found in Thoen *et al.* (122) and Thorel *et al.* (123).

Natural and Experimental Hosts

Birds

All species of birds can be infected with *M. avium*. Generally speaking, domesticated fowl or captive wild birds are affected more frequently than those living in a wild state. Avian tuberculosis has been reported in domesticated or captive-raised ducks, geese, swans, peafowl, pheasants, quail, partridge, pigeons, doves, turkeys, birds of prey, and other captive and/or wild birds. Pet birds including parrots, cockatoos, budgerigars, finches, flycatchers, and canaries have been infected (30, 34, 78, 94, 97).

Although uncommon, infections and disease may be expected to develop in wild birds in contact with farm premises where avian tuberculosis is prevalent in chickens. Pheasants seem to be unusually susceptible to infection by *M. avium* (97). The disease has also been observed in sparrows, crows, barn owls, cowbirds, blackbirds, eastern sparrow hawks, starlings, wood pigeons, Canada goose, wild turkey (36), American bald eagle (44), painted quail (75) and sandhill and whooping cranes.

Avian tuberculosis has been reported in ostriches, emus, and rheas housed in zoologic parks. Recently, avian tuberculosis was diagnosed in a 3-year-old female emu in a commercial flock (95).

Avian tuberculosis, although reported (43), is not common in turkeys and usually is contracted from infected chickens. Avian tuberculosis has been reported in wild birds and is reviewed elsewhere (9, 30, 34, 50, 54, 70, 91).

Avian tuberculosis is more common among birds in many zoologic gardens than in domestic fowl (74). Infections usually result from *M. avium* serovar 1 or serovar 2 (116). Tuberculosis in psittacine birds may also be due to *M. tuberculosis* or *M. bovis* (1, 47, 78). Recently, it was found that during a 9-year period of identification of *Mycobacterium* sp. infected pet birds in

Table 23.5. Comparative pathogenicity of *Mycobacterium avium* for certain mammals.

Animal	Susceptibility
Cat	Highly resistant
Cattle	Infection occurs; usually localized
Deer	Infection reported
Dog	Highly resistant
Goat	Assumed to be relatively resistant
Guinea pig	Relatively resistant
Hamster	Susceptible (intratesticular)
Horse	Infection reported
Llamas	Susceptible
Marsupial	Infection reported
Mink	Readily infected
Monkey	Susceptible
Mouse	Relatively resistant
Rabbit	Readily infected
Rat	Relatively resistant
Sheep	Moderately susceptible
Swine	Readily infected

Switzerland that *M. genavense* (71.8%) was the predominant species followed by *M. avium* complex (16.7%), *M. fortuitum* (4.2%), *M. tuberculosis* (4.2%), and *M. gordonae* (2.2%) and *M. nonchromogenicum* (2.2%) (46). In other studies (82), *M. genavense* is a common isolate of zoological collections. Luckily, *M. genavense* may be differentiated from *M. avium* by polymerase chain reaction with restriction enzyme digestion (72).

Mammals

M. avium can infect and cause disease in some domesticated mammals, but lesions usually are localized (33, 34, 40, 66, 112). Microorganisms may multiply in tissue for a considerable period and induce sensitivity to tuberculin. Disseminated tuberculosis caused by *M. avium* has been reported in rabbits and swine (111).

Although spontaneous infection of mammals may not be of comparable severity to that developed in fowl, it is possible to produce extensive changes in many species of mammals by introducing the infective agent artificially. The relative pathogenicity of *M. avium* for many of the domesticated mammals is summarized in Table 23.5.

In the United States and Europe, *M. avium* serovar 2 is the most common cause of tuberculous lesions in swine (52, 109, 130). Tuberculosis will remain an unnecessary economic burden on the swine industry until it is eliminated from chickens and other barnyard fowl. There has been a gradual but definite decrease of tuberculosis in swine in the United States (114). Reasons for the decrease may be the lower incidence of avian tuberculosis in poultry as a result of the increasing practice of maintaining one-age flocks and a change to confinement rearing of swine. In the past when pork prices were high, pork production would expand by using vacant farm buildings (outdated chicken houses). With the advent of contract pork production, a market no longer exists for swine raised in this fashion.

Age of Host Commonly Affected

Avian tuberculosis appears to be less prevalent in young fowl not because the younger birds are more resistant to infection, but because in older birds the disease has had a greater opportunity to become established through a longer period of exposure. Although tuberculosis lesions are usually less severe in young chickens than adult birds, extensive or generalized avian tuberculosis in young chickens has been observed. Such birds are an important source of dissemination of virulent tubercle bacilli and must be considered a source to other fowl and susceptible mammals.

Tuberculosis causes important death losses in captive wild birds of zoo aviaries (74). The significance of these findings is emphasized by reports of disease in valuable endangered species. Numerous reports are also available on tuberculosis in pet birds (1, 12, 46, 78, 95, 100, 126).

Transmission

The tremendous number of tubercle bacilli exuded from ulcerated tuberculous lesions of the intestine in poultry creates a constant source of virulent bacteria. Although other sources of infection exist, none equals infective fecal material in the importance for dissemination of avian tuberculosis. Fecal discharges may also contain tubercle bacilli from lesions of the liver and mucosa of the gallbladder expelled through the common bile duct. The respiratory tract is also a potential source of infection, especially if lesions occur in tracheal and bronchial mucosa.

The contaminated environment, especially soil and litter, is the most important source for the transmission of the bacilli to uninfected animals. The longer the premises have been occupied by infected birds and the more concentrated the poultry population, the more prevalent the infection is likely to be.

M. avium may persist in soil for up to 4 years (92). *M. avium* bacilli remained viable in carcasses buried 3-feet deep for 27 months. Virulent strains of *M. avium* have been found to survive in sawdust for 168 days at 20°C and 244 days at 37°C (93).

M. avium has been isolated from eggs of naturally infected chickens, but hatched chicks failed to develop avian tuberculosis (30, 32, 34, 92). *M. avium* does not survive in eggs after 6 minutes of boiling, and in preparation of scrambled eggs, 2 minutes of frying was sufficient to kill the bacteria.

M. avium can be disseminated in carcasses of tuberculous fowl and offal from chickens dressed for food. Cannibalism might play a part in transmission.

Dissemination of *M. avium* on shoes and equipment used in the care and maintenance of infected poultry (crates and feed sacks) can be involved in transfer from diseased to healthy flocks.

Wild birds such as sparrows, starlings, and pigeons may be infected with *M. avium* and may spread *M. avium* to poultry flocks. Although not very likely, swine may have ulcerative intestinal lesions from *M. avium* and, thus, constitute a source of infection for other animals and birds.

Clinical Signs

Clinical signs are not pathognomonic. In advanced infections, the bird will be less lively than its pen mates, will fatigue easily, and may be depressed. Although appetite usually remains good, pro-

gressive and striking loss of weight commonly occurs, evident as atrophy of breast muscles with a prominent keel. In extreme cases, the body fat eventually disappears, and the face of the affected bird appears smaller than normal.

Feathers assume a dull and ruffled appearance. Comb, wattle, and earlobes often appear pale and thinner than normal and have a dry epidermis. Occasionally, the comb and wattles have a bluish discoloration. Icterus, indicative of advanced hepatic damage, may be noted.

Even when the disease is severe, the temperature of the affected bird remains within the normal range. In many instances, the bird reveals a unilateral lameness and walks with a peculiar jerky hopping gait. Lesions may rupture and discharge fluid with caseous material. Paralysis from tuberculous arthritis can sometimes occur.

With advanced emaciation, nodular masses can be palpated along the intestine. However, the hepatomegaly that many tuberculous birds possess may make this procedure difficult or impossible. Intestinal nodules may be ulcerative, resulting in severe diarrhea.

Affected birds may die within a few months or live for many, depending on severity or extent of the disease. A bird may die suddenly as a consequence of hemorrhage from the rupture of the affected liver or spleen.

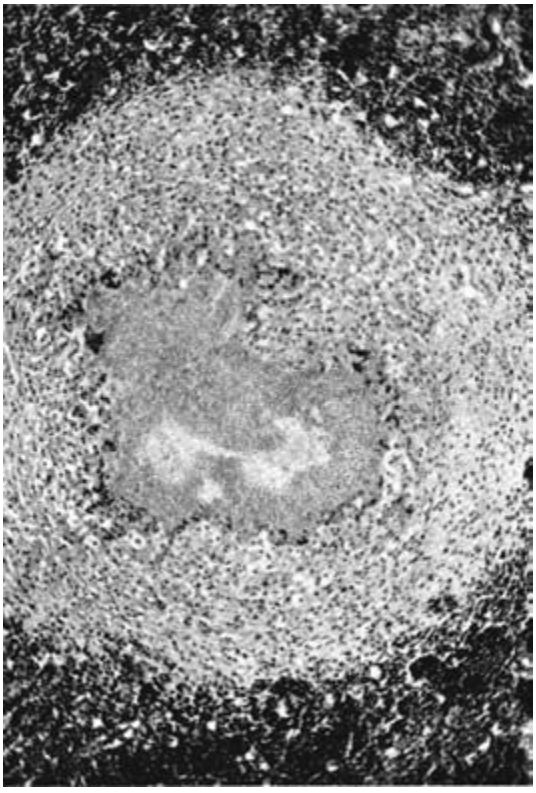
In a flock situation, clinical signs may vary between infected birds. Two clinical syndromes were described in an infected commercial flock of laying hens. One group had good body condition, continued to lay eggs but had nodular masses in the infraorbital sinuses, liver, and intestines. Another group within the same flock was emaciated, did not lay eggs, lacked sinus lesions, and had numerous nodular masses in internal organs (38).

Pathology

Gross

Lesions are seen most frequently in liver, spleen, intestines, and bone marrow. Some organs, such as heart, ovaries, testes, and skin, are affected infrequently and cannot be considered organs of predilection. For turkeys, ducks, and pigeons, lesions predominate in the liver and spleen but occur also in many other organs (34).

Lesions of avian tuberculosis in chickens are characterized by pinpoint to several centimeter, irregular grayish yellow or grayish white nodules in spleen, liver, and intestine (Fig. 23.18A, B, D). Involvement of liver and spleen results in enlargement, which can result in fatal hemorrhage from rupture. Large nodules have an irregular knobby contour, with smaller nodules present over the capsular surface of affected organs. Lesions near the surface in such organs as liver and spleen are easily enucleated from adjacent tissues. Nodules are firm but can be incised easily. Mineralization is rare. On cross-section, a nodule may contain a variable number of small yellowish foci or a single soft yellowish caseous center surrounded by a fibrous capsule. The capsule continuity may be interrupted by small circumscribed necrotic foci. The fibrous capsule is of variable thickness and consistency, depending on the size and duration of the lesion. It is barely discernible or apparently absent in small lesions and measures 1–2 mm in thickness in larger nodules. Intestines may have white firm nodules that bulge from the serosal surface. Involvement of lungs is usually less severe than that of liver or spleen.



23.19. Small tuberculous granuloma in bone marrow of a naturally infected chicken. Central necrotic region is surrounded by a zone of dense connective tissue. ×100.

Granuloma formation is frequent within bone marrow (Figs. 23.18C and 23.19). Infection of bone marrow probably occurs very early in the course of the disease and results from the bacteremia.

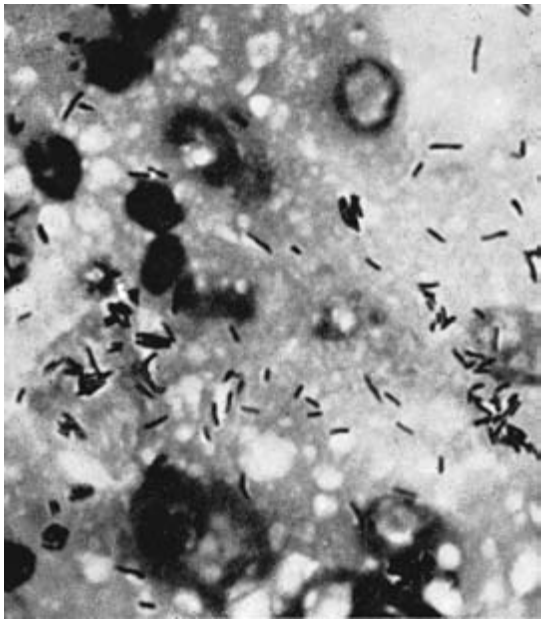
Microscopic

The basic lesion of *M. avium* infection consists of multiple granulomas with a central caseous necrosis. Granulomas consist of the accumulation of large number of macrophages with abundant cytoplasm (epithelioid macrophages). Epithelioid macrophage populations expand within the granuloma and fuse near the periphery to form multinucleate giant cells. In larger nodules, the central area of the granuloma may have coagulative or caseous necrosis. In large nodules, only the multinucleate giant cells may persist as a mantle around the necrotic core. Immediately peripheral to the multinucleate giant cells is a collection of both epithelioid and histiocytic macrophages (Fig. 23.20). A fibrous capsule consisting of fibrocytes and minute blood vessels also occurs near the outer portion of the peripheral area. Acid-fast bacilli are numerous in the central or necrotic zone of the tubercle but can be found in large numbers in the epithelioid zone adjacent and distal to multinucleate giant cells (Fig. 23.21).

The outermost region of the granulomas is encapsulated by fi-



23.20. Developing tubercle in lung of chicken. ×100.



23.21. Numerous tubercle bacilli in smear preparation from a small lesion of lung of a naturally infected chicken. Ziehl-Neelsen stain, ×1600.

brous connective tissue, macrophages, some lymphocytes, and an occasional granulocyte. Calcification of the tubercle rarely occurs in fowl. Amyloid deposition in the surrounding parenchymal elements has been reported in liver, spleen, and kidney.

Microscopically, lesions of avian tuberculosis in turkeys vary considerably but are similar to avian tuberculosis of chickens. In other instances, lesions are diffuse, with extensive destruction of surrounding parenchyma. Cytoplasmic masses or large giant cells may be numerous, and large numbers of granulocytes are commonly present. Some lesions become circumscribed by a broad, dense zone of fibrous connective tissue.

Pathogenesis of the Infectious Process

Ingestion of the bacillus results in intestinal infection and eventual bacillemia. Bacillemia allows for the transfer of bacilli from the intestine to the liver directly. The bacillemia, which probably occurs intermittently and perhaps early in most instances, also provides for a generalized distribution of lesions. No tissue, with the possible exception of the central nervous system, appears to be exempt from infection.

Cheville *et al.* (17) studied experimental *M. avium* infection in chickens. The disease course in young chicks lasted for 30 days after intravenous challenge. Single acid fast bacilli were first found 5 days postinfection in cells of periarterial lymphoid sheaths of the spleen without other histologic evidence of infection. Many bacilli were found within aggregated histiocytic macrophages of the sheath 10 days postchallenge. By day 14, miliary tubercles were found within lymphoid sheaths. Delayed type hypersensitivity (DTH), as judged by wattle thickness, first occurred 2 days postinfection and increased in intensity as the disease progressed. This response decreased as the disease became more severe. The disease process was divided into 3 periods, a latency period, a lesion development period, and a cachexia period. The latency period occurred for the first 7 days of the infection. During this period, there were no microscopic lesions, but DTH reactions appeared and increased in intensity with time. The lesion development period occurred from days 8–17 postinfection. Bacilli multiplied in lymphoid sheaths during this time. Serum antibody titers developed; the thymus atrophied; and small tubercles with few bacilli developed. Cachexia lasted from day 18 until death. During this period, massive tubercles with large numbers of bacilli developed; there was lymphoid atrophy; DTH disappeared; and amyloid was deposited at the periphery of tubercles. In addition to lymphocyte-intact chickens, these studies also used both bursectomized and thymectomized chicks. There was very little difference in the pathogenesis between lymphocyte-intact and -depleted chicks.

The capacity of *M. avium* to produce progressive disease may be related to cell wall constituents and certain complex lipids present in the cell wall, such as cord factor, sulfur-containing glycolipids (sulfatides), or strongly acidic lipids (88, 115). *M. tuberculosis* and *M. avium* prevent fusion of phagosomes (the vacuole where they reside intracellularly) with lysosome and maturation of the resulting phagolysosome (103). It appears, however, that the effect of the aforementioned components alone or together on phagosome-lysosome fusion cannot account for virulence. DTH develops following exposure to mycobacteria; once activated,

macrophages demonstrate an increased capacity to kill intracellular *M. avium*. The DTH responses are mediated by lymphocytes, which release lymphokines that act to attract, immobilize, and activate blood-borne mononuclear cells at the site where virulent bacilli or their products exist. Tumor necrosis factor, alone or in combination with interleukin-2, but not γ interferon, has been associated with macrophage killing of *M. avium* serovar 1 (7). The DTH contributes to accelerated tubercle formation and is, in part, responsible for cell-mediated immunity in tuberculosis. Activated macrophages that lack sufficient subcellular microbiocidal components to kill virulent tubercle bacilli are destroyed by the intracellular growth of the organism, and a lesion develops. A combination of toxic lipids and factors released by virulent *M. avium* may 1) cause disruption of the phagosome, 2) inhibit phagolysosome formation, 3) interfere with the release of hydrolytic enzymes from the attached lysosomes, and/or 4) inactivate lysosomal enzymes released into the cytoplasmic vacuole. Toxic oxygen metabolites are not responsible for killing activated macrophages. However, the significance of hydrogen peroxide, activated oxygen radical(s), and nitric oxide in resistant macrophages of birds exposed to virulent *M. avium* remains to be elucidated (115). More recently, *M. avium* has been shown to induce caspase-1 activity in macrophages and may serve as a mechanism for its pathogenicity (96).

Diagnosis

A presumptive diagnosis of avian tuberculosis in fowl usually can be made based on gross lesions (15). Demonstration of acid-fast bacilli in smears or histologic sections of liver, spleen, or other organs strengthens the diagnosis and is sufficient for most diagnostic cases. Inoculation of suitable media to isolate and identify the causative agent confirms the diagnosis of avian tuberculosis and allows speciation of the causative agent (57). In live, suspected infected birds, fecal smears for culture, staining, and/or PCR may be attempted but these tests are not reliable due to intermittent or no fecal shedding of bacilli. (124) Fecal positivity increases as the disease course progresses (106). Polymerase chain reaction has been used to detect mycobacteria including *M. avium* and *M. genavense* in formalin-fixed tissue which may further aid diagnostic considerations (41).

Tuberculin Test

When administered properly, the tuberculin test provides a satisfactory procedure for determining presence of avian tuberculosis in a flock. The technique involves intradermal injection of the wattle with 0.03–0.05 mL of a U.S. Department of Agriculture (USDA) supplied purified protein derivative tuberculin prepared from *M. avium* in a manner previously described (3). The injection site then is monitored for a reaction. A more complete methodology and interpretation has been reported elsewhere (112). Tuberculin testing in poultry may reveal a false-negative result twice during the course of infection: once during early infection and again during late infection, when there is immune system exhaustion or anergy. This test is also unreliable in some bird species (104).

Serology

Enzyme-linked Immunosorbent Assay

Enzyme-linked immunosorbent assay (ELISA) has detected mycobacterial antibodies in sera of chickens experimentally inoculated with *M. avium* serovar 2, but false-positives may be common (120). ELISA is less specific than the tuberculin test.

Rapid Agglutination Test

A whole-blood agglutination test has been described for diagnosis of avian tuberculosis in fowl (55). The agglutination test has been more useful for detecting infected birds in a diseased flock; however occurrence of false-positive agglutination reactions in healthy birds is a drawback (42).

Differential Diagnosis

The most expedient way to diagnose the disease is by necropsy. Granulomas are rather characteristic, but other conditions must be differentiated. These include coligranulomas (Hjarre's disease), pullorum disease, other *Salmonella* infections, *Staphylococcus* infection, fowl cholera, aspergillosis, and neoplasia. Presence of numerous acid-fast bacilli in lesions is significant. When available, culture and identification of *M. avium* is helpful but not essential for a diagnosis.

Intervention Strategies

Management Procedures

In backyard poultry and captive birds, the tuberculin test should be used to detect avian tuberculosis. Removal of chickens that react reduces environmental contamination and subsequent infections. The whole-blood agglutination test also may serve to detect infected birds and may be more reliable. However, if the residual flock is permitted to occupy the same contaminated premises, contaminated soil may be a continuing source for infection. Furthermore, neither the tuberculin nor the agglutination tests can be depended upon for detection of every tuberculous fowl. As long as one infected bird remains in a flock, dissemination of the disease to healthy fowl is possible. Consequently, termination of the entire flock and repopulation on noninfected soil may be the best approach to control avian tuberculosis.

Procedures for establishing and maintaining avian tuberculosis-free backyard flocks should include the following: 1) Abandon old equipment and establish other facilities on new soil. Ordinarily, it has been impractical to render an infected environment satisfactorily safe by disinfection. 2) Provide proper fencing or other measures to prevent unrestricted movement of chickens, thus preventing exposure from previously infected premises. 3) Eliminate the old flock, burning carcasses of birds that show lesions of tuberculosis. 4) Establish a new flock in the new environment from avian tuberculosis-free stock. If chickens in a clean flock are prevented access to an infected environment and are protected against accidental exposure to an infected environment and accidental exposure to *M. avium*, it is reasonable to believe that they will remain free from avian tuberculosis.

Recommendations for control of avian tuberculosis in exotic birds include the following: 1) Prevent contact with tuberculous

birds; premises and housing previously used by them are to be avoided. 2) Quarantine additions to the aviary for 60 days and retest with avian tuberculin.

Vaccination

Use of experimental vaccines containing inactivated and/or live mycobacteria for protecting chickens against tuberculosis has been evaluated (87). The best results were obtained in chickens vaccinated with live *M. intracellulare* serovar 6 (*M. avium* serovar 6) given orally. These fowl showed 70% protection after intramuscular (IM) challenge with *M. avium*. Encouraging results were also reported in chickens after combined IM vaccination with inactivated plus live *M. intracellulare* serovar 7 and serovar Darden (*M. avium* serovars 7 and 19). Recently, vaccination of chickens using various fractions of a homologous strain of *M. avium* for vaccine production and challenge revealed that the number of lesions and bacilli per gram of liver were decreased; it did not however prevent infection (29).

Treatment

Treatment with antituberculosis drugs is impractical and is rarely done to treat domestic backyard poultry. However, combinations of antituberculosis drugs have been used to treat certain exotic birds maintained in captivity (126). Clinical remission was observed in 3 birds that received a combination of isoniazid (30 mg/kg), ethambutol (30 mg/kg), and rifampicin (45 mg/kg). The recommended duration of therapy was 18 months, provided that there were no adverse side effects. Additional investigations are needed to develop suitable regimens for the treatment of tuberculosis in various exotic birds.

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Other Bacterial Diseases

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Miscellaneous and Sporadic Bacterial Infections

A number of surveys of bacteria isolated from poultry have been done, which often include a variety of unusual bacteria of low incidence. For example, bacteriologic examination of clinically ill broilers within 2 weeks of processing resulted in 132 isolates. The majority of these were various species of staphylococci and *E. coli*, but *Corynebacterium, Stomatococcus, Micrococcus, Lactobacillus, Moraxella, Proteus, Acinetobacter, Pseudomonas,* and *Yersinia* were also isolated (12). Similarly, microbial surveys of eggs, dead embryos, chicks with omphalitis and yolk sac infections, and chick mortality often reveal a diversity of unusual bacteria, generally in low incidence that are found in the environment and are not normally associated with disease. This is especially true for commercially raised ratites (48, 143). As the significance of the isolates that are recovered infrequently in surveys is unknown or not considered significant, they have not been covered in detail in this review. Bacteria recovered from environmental sampling and organisms considered to be normal flora also have not been included.

Interest in achieving and maintaining intestinal homeostasis in meat-producing birds has led to methods to quantify microbial populations by molecular methods (36, 217, 270). Organisms identified in these studies generally are not discussed in this review.

Acinetobacter

Acinetobacter is a nonfermentative, strictly aerobic, small, gram-negative coccobacillus in the family Moraxellaceae (181). It is a common organism in poultry environments and has been recovered from the eyes of normal ducks (55) and respiratory tracts of healthy chickens (7). It is occasionally recovered from dead-in-shell embryos and weak chicks (130, 155, 220). *A. lwoffi* and *A. calcoaceticus* were isolated from outbreaks of septicemia in hens. Mortality was approximately 15%, and there was multifocal necrosis and green discoloration of the liver (89, 132). Turkeys are also affected as *Acinetobacter* has occasionally been isolated from dead-in-shell embryos and weak poults, respiratory disease, septicemia, and inflamed joints of turkeys (91). Other clinical presentations include pigeons with arthritis (85) and ducks with arthritis, septicemia, or airsacculitis (33, 262).

Actinobacillus/Gallibacterium

Bacteria previously identified as *Actinobacillus salpingitidis*, avian *Pasteurella haemolytica*-like, or *P. anatis* have been reclassified and placed into a new genus *Gallibacterium* (59). The organisms may colonize avian hosts and cause no clinical disease, or cause septicemia, respiratory disease, severe conjunctivitis, or salpingitis (73, 93, 159, 173, 180, 236). Chickens, ducks, geese, and ostriches have been affected. Field isolates from septic laying hens and a well-characterized strain of *G. anatis* were pathogenic for pullets and layers following inoculation (38, 237, 238). Mortality was higher in birds inoculated intravenously compared to those inoculated intraperitoneally, and lesions were more severe in birds that had been experimentally immunosuppressed before exposure (38).

Atypical *A. lignieresii*, currently identified as taxon 2 and taxon 3, was isolated from lesions of salpingitis in egg-laying ducks and geese. Isolation of these organisms from the cloaca and penis of normal geese suggests that salpingitis probably results from an ascending infection (35). A similar conclusion was reached about the role of *Actinobacillus* in goose venereal disease (see below) (161). Severe conjunctivitis resulting in blindness in a free-living Canada goose was caused by *A. suis* (159).

Actinomyces/Arcanobacterium (Corynebacterium)

A chronic, disseminated granulomatous disease of turkeys in Canada suspected to be actinomycosis has been observed sporadically since 1955 (219). Serious outbreaks of osteomyelitis involving the proximal tibia, thoracic vertebra, and/or proximal tibiotarsi caused by *Arcanobacterium* (*Actinomyces*) *pyogenes* in commercial male turkey flocks resulted in considerable economic loss (41). Lame birds in 20 affected flocks averaged 20% (range, 5–50%), age averaged 16-weeks (range, 12–20 weeks), and weekly mortality averaged 2.8% (range, 0.5–10.5%). Hen flocks were not affected (19). Club-shaped, pleomorphic, gram-positive bacilli in smears of lesions provided a rapid diagnosis (41). Biochemical and serologic evaluation of isolates from 9 flocks indicated they were either identical or very closely related. An agar-gel precipitin test was highly effective at detecting antibodies (19). Treatment of an affected flock with penicillin in the feed (100 g/ton) resulted in a gradual improvement in the flock after 8–10 days on medication. Osteomyelitis was reproduced in 15-week-old male turkeys inoculated intravenously with a representative isolate.

Septicemia, visceral lesions, cutaneous abscesses, mortality of nearly 14%, and a decrease in egg production of over 27% occurred in caged layers infected with *A. pyogenes*. Portal of entry was through skin lesions caused by poor caging (71).

A. pyogenes was isolated from abnormal kidneys of broilers, but attempts to reproduce renal lesions with the isolates were not successful (228).

Aegyptianella

Aegyptianella are obligate intracellular organisms in the family Anaplasmataceae and are most closely related to *Anaplasma* spp. (208). They cause the tick-borne disease aegyptianellosis (105). The disease has been identified in a variety of birds in-

cluding chickens, turkeys, and guinea fowl. Affected birds experience increased mortality and develop severe anemia, which can predispose them to ascites and right ventricular heart failure (121). Aegyptianellosis occurs mainly in free-ranging poultry and wild birds that are infested with fowl ticks in the genus *Argas*. With the exception of wild turkeys in the Rio Grande area of Texas (51) and an Amazon parrot imported into England from South America (197), the organism has only been identified in Europe, Asia, and Africa. Diagnosis depends on identifying the typical organism in erythrocytes of infected birds (105). Treatment with tetracyclines and supportive care are generally effective (105). Prevention is the same as that for spirochetosis (see *Borrelia* below).

Aerobacter

Aerobacter has been recovered occasionally from dead embryos (131). Twenty percent mortality occurred in a flock of turkeys infected with *Aerobacter (Klebsiella) aerogenes*. Affected poults had enlarged mottled livers, swollen kidneys, and visceral urate deposits ("gout"). Intramuscular injections of 15–20 mg/kg polymyxin B produced transient coma-like signs but were successful in stopping the disease (110).

Aeromonas

Aeromonas is commonly found in aquatic environments and animals. It is a frequent intestinal inhabitant of poultry that can easily contaminate carcasses during processing. Intestinal colonization is dependent on the O-antigen lipopolysaccharide of the organism (168). *Aeromonas* has public health significance as a potential cause of food-borne illness because of the production of cytotoxins (23).

Aeromonas hydrophila, either alone or in combination with other organisms, can cause localized and systemic infections in avian species including poultry (101, 223). High numbers were found in chickens with watery mucoid feces (87). *Aeromonas* was recovered from turkeys experiencing severe diarrhea. Inflammation and hemorrhage of the intestinal mucosa were characteristic findings in affected poults. Experimental inoculation of chicks with the turkey isolate caused significant mortality (97). *Aeromonas* was among the organisms identified from cellulitis lesions in turkey carcasses at processing (189). *A. hydrophila* has been isolated from ducks with salpingitis (35), septicemia (154), airsacculitis (262), and granulomatous inflammation of salt glands (137). A bacterin prepared from 3 strains that caused high mortality in experimentally inoculated ducklings successfully controlled losses in commercial duck flocks (154). *A. formicans* has been isolated infrequently from arthritic lesions in ducks at processing (33). *Aeromonas* and *E. coli* were the most frequently isolated bacteria from geese with necrotic inflammation of the phallus (see Goose Venereal Disease below) (163).

Aeromonas is among environmental bacteria that can be recovered from dead-in-shell embryos and weak chicks (155). Microbial contamination of ostrich eggs by *Aeromonas* was associated with reduced hatchability (80).

Arcobacter

Bacteria in the genus *Arcobacter* previously were classified as campylobacters (see chapter 18) to which they are closely related. *Arcobacter* differ from campylobacter in their ability to grow aerobically and at 15°C (191). *Arcobacter* species have public health significance as a cause of food-borne illness, but produce no recognized clinical disease in experimentally exposed chickens or turkeys (150). Usually it is isolated from carcasses or retail meat, however, the organism has been recovered from cloacal swabs of chickens and from the ceca of ducks (10, 129, 207, 264). Special procedures and media can be used to selectively isolate the organism (119). Molecular methods have been developed to rapidly detect, speciate, and genotype organisms. Ducks are more frequently affected than chickens or turkeys, but a high rate of broiler carcass contamination has been identified (11).

Bacillus

Bacillus spp. occasionally have been associated with embryo mortality and yolk sac infections in chickens (42, 58, 253), turkeys (43), ducks (25, 220), and ostriches (79, 80). *Bacillus* spp. and *E. coli* were the most commonly cultured bacteria from reproductive disorders of hens (104). *B. cereus*, an organism that can cause food-borne illness in people, infected turkey hens following artificial insemination and was found in 25% of their unhatched eggs. The prevalence fell to 4% after the infection was controlled (43). Certain strains of *Bacillus* interfere with intestinal colonization of enteric pathogens and have value as probiotics (18, 142).

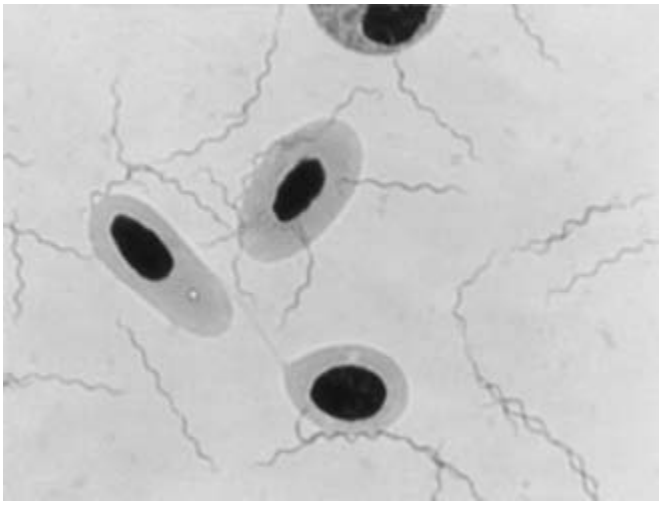
Keratinases (subtilisins) produced by *B. licheniformis* have the ability to degrade feathers (90). Feather degradation is associated with focal ulcerative dermatitis of turkey breast skin but a correlation between keratinase exposure and lesion formation has not been investigated (22).

Bacteroides

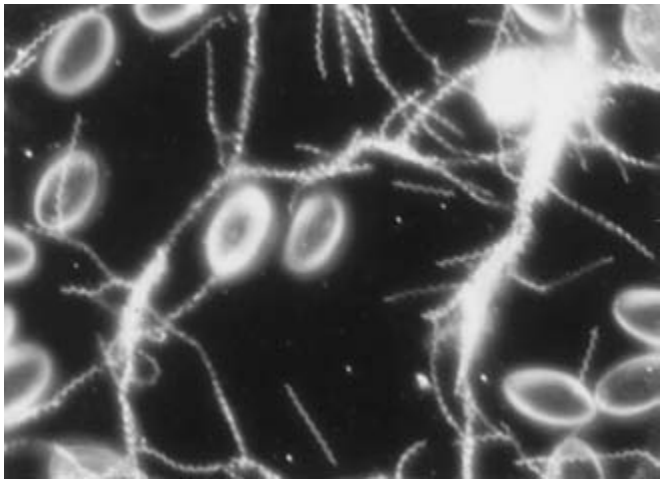
Bacteroides spp. are anaerobic, non-sporeforming, gram-negative rods that are normally found in high numbers in the lower digestive tract, especially ceca, of poultry. They are rarely associated with disease. *B. fragilis* occasionally has been isolated from the oviduct of laying hens experiencing salpingitis (34). *Bacteroides* also has been associated with inflammation of the phallus and multifocal liver necrosis in geese (see Goose Venereal Disease below) (29, 76).

Bilophila

Bilophila is commonly found in the large intestine of animals, including human beings, and has been associated with appendicitis and localized inflammatory lesions. Examination of lower intestinal contents from broiler chickens in the southeastern US did not find an association between malabsorption syndrome and the bacterium (166).



23.22. *Borrelia anserina* in blood film during the acute stage of infection. Giemsa, ×1200.



23.23. *Borrelia anserina* in plasma from infected chicken during terminal stages of spirochetosis. Note the agglomeration of organisms. Dark-field, ×480.

Borrelia

Borrelia anserina causes a tick-borne, non-relapsing borreliosis (spirochetosis) in avian species including chickens, turkeys, pheasants, geese, and ducks. The disease is usually an acute septicemia characterized by variable morbidity and high mortality, but may also be mild when birds are infected with low virulent strains (20, 227). Birds also can develop asymptomatic infections with *B. burgdorferi*, the cause of Lyme disease in people, and serve as hosts for ticks capable of spreading the spirochete to mammals (64, 99, 133, 160).

Occurrence of spirochetosis corresponds with the subtropical and tropical distribution of fowl ticks in the genus *Argas*, which serve as both the reservoir and primary vector. Attempts to transmit *B. anserina* with *Amblyomma cajennense* were unsuccessful (144). Occasional outbreaks have been identified in the southwestern United States in chickens, turkeys, and pheasants (70). Extensively reared free-range flocks are more likely to be affected than confined flocks, and indigenous breeds of chickens are generally more resistant than exotic breeds (203). In addition to ticks and other biting arthropods (mosquitoes, mites), infection can result from cannibalism, scavenging on carcasses, multiple use of syringes and needles, or ingestion of infective blood, droppings, or infected ticks. Virulent strains are capable of penetrating unbroken skin. *B. anserina* is not resistant outside of the host. Recovered birds are not carriers; organisms disappear from tissues at or shortly after they disappear from the circulation (20).

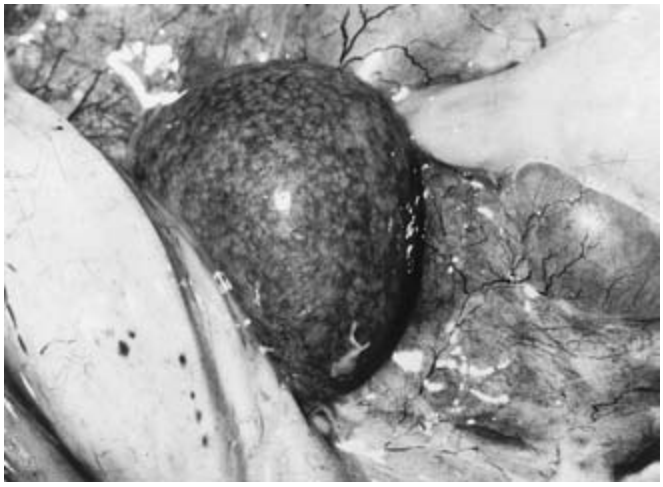
Borrelia anserina is a highly motile, helical bacterium (Fig. 23.22) that stains well with aniline dyes, Romanowsky-type stains, and silver impregnation. Spirochetes can be readily identified in wet smears of blood or tissues by dark-field or phase microscopy (Fig. 23.23).

Birds infected with virulent strains of *B. anserina* are visibly sick, with cyanosis or pallor of the comb and wattles, ruffled feathers, dehydration, inactivity, and anorexia. There is a marked elevation in body temperature that begins shortly after infection and a rapid loss of body weight. Affected birds pass fluid, green droppings containing excess bile and urates, and have increased water consumption. Late in the disease, birds develop paresis or paralysis, become anemic, and are somnolent to comatose. Body temperatures are subnormal just prior to death. Birds recovering from the disease are often emaciated and have temporary residual weakness or paralysis of one or both limbs (20). Infection with low virulent strains may be mild or inapparent (70).

Marked enlargement and mottling of the spleen is typical of spirochetosis (Fig. 23.24) but may not be evident if birds are infected with low virulent strains (70) or early in the disease. Livers often are enlarged and contain small hemorrhages, pale foci, or marginal infarcts. Kidneys are swollen and pale with excess urates distending the ureters. Green, mucoid intestinal contents are usually present, and there often are variable amounts of hemorrhage, especially at the proventriculus-ventriculus junction. Fibrinous pericarditis occurs infrequently. Extensive hemorrhage and muscle necrosis have been reported in naturally infected pheasants (20).

Splenic lesions result from macrophage and lymphoid hyperplasia, erythrophagocytosis, and hemosiderin deposition. Multifocal necrosis and hyalinization of white pulp and/or extensive hemorrhage may be present in some birds. The liver is congested with increased periportal infiltrates of mixed lymphocytes, hemocytoblasts, and phagocytic cells with vacuolated cytoplasm. Erythrophagocytosis and hemosiderin are seen in Kupffer cells. Extramedullary hematopoiesis may be present. Lymphoplasmacytic infiltrates occur in kidneys and intestinal lamina propria of some birds. Occasionally, there is a mild to moderate lymphocytic meningoencephalitis (16, 20).

Spirochetosis can be diagnosed by finding characteristic lesions in birds with signs consistent with the disease. Larval ticks on the birds, evidence of tick bites, or presence of ticks in



23.24. An enlarged, mottled spleen is characteristic of spirochetosis caused by highly virulent strains of *Borrelia anserina* in chickens. Low virulence strains may not produce splenic lesions, and they may appear differently in other avian species depending on the amount of necrosis and hemorrhage. A few serosal hemorrhages on the proventriculus also can be seen in this bird.

the bird's environment increases the likelihood of spirochetosis. The disease is confirmed by demonstrating *B. anserina* or its antigens in affected birds or by seroconversion following recovery. Dark-field microscopy is the method of choice for identifying spirochetes in blood (21). In tissue sections, spirochetes can be demonstrated by silver impregnation staining procedures (70).

Borrelia cannot be cultured on routine bacteriologic media but will grow in chick embryos following yolk sac inoculation or in susceptible chicks (21). It can be grown in liquid medium but loses virulence (152). Bursectomy or dexamethasone treatment of chicks may be necessary for low virulent strains to grow to detectable numbers (78). Strains are usually maintained in ticks, day-old chicks, chicken or turkey embryos, or by cryopreservation at –70°C in 5% glycerol or dimethylsulfoxide added to infective blood (21, 145). Several serologic methods have been used to detect antibodies in immune birds including serum plate agglutination, slide agglutination, spirochete immobilization, agar gel precipitin, and indirect fluorescent antibody tests. Spirochetal antibodies readily can be detected in yolk of eggs from immune hens (21).

Arsenicals and most antibiotics, including penicillin, chloramphenicol, kanamycin, streptomycin, tylosin, and tetracyclines, are effective in treating infected birds. Intramuscular injections of penicillin at 20,000 IU/bird given three times in 24 hr or 20 mg oxytetracycline given daily for two days represent current treatment regimens (20).

Active immunity follows recovery or immunization. Immunity is serotype-specific; infection with other *B. anserina* serotypes can occur in recovered or vaccinated birds. An autogenous or polyvalent vaccine containing multiple serotypes may be necessary to provide full protection (254). Controlled infection followed by antibiotic treatment 3 days later also has been used to induce active immunity. Passive maternal immunity provides protection for 5–6 weeks and hyperimmune serum protects birds against challenge for up to 3 weeks (20). Antibodies prepared against an outer surface lipoprotein provide passive homologous but not heterologous protection (213).

Preventing fowl tick infestation is the best method to control spirochetosis in endemic areas. Young chickens in dense poultry areas during the summer were more likely to be infested with fowl ticks (222). Adult ticks can remain alive without feeding and carry the spirochete for as long as 3 years (20).

Citrobacter

Citrobacter is a genus in the Enterobacteriaceae family. The organism commonly colonizes mucous membranes of the respiratory and digestive tracts of normal birds, but can be an opportunistic pathogen. *Citrobacter* is one of many environmental bacteria that are occasionally isolated from unhatched eggs, weak chicks, and yolk sac infections (155, 253). It has been isolated from the liver of two-week-old turkey poults with respiratory disease (91). Antimicrobial testing of 37 isolates from diseased or dead turkey poults between one and 35 days of age indicated sensitivity (MIC_{50}<1µg/ml) to enrofloxacin, ceftiofur, gentamicin, and trimethoprim/sufadiazine (212). *C. freundii* infrequently has been isolated from young ducks with salpingitis (35).

Coenonia

Coenonia is a recently described genus that contains a single species, *C. anatine*, previously identified as taxon 1502. It is a *Riemerella anatipestifer*-like bacterium that causes a similar exudative septicemia in ducks and geese (252).

Coryneform Bacteria

A gram-positive rod with characteristics of *Erysipelothrix*, *Lactobacillus*, and *Listeria* was isolated from an outbreak of polyarthritis in chickens (174). *Corynebacterium* sp. accounted for 18% of the 132 isolates from blood samples, livers, and hock joints of clinically ill commercial broilers within 2 weeks of processing (12).

Enterobacter/Pantoea

Enterobacter is a normal inhabitant of the avian digestive tract (30). Similar to other gram-negative bacteria in the Enterobacteriaceae family, it can infect eggs and young birds causing embryo loss, omphalitis, yolk sac infections, and mortality in young birds (91, 136, 155, 212, 253, 263). *Enterobacter* was isolated infrequently from turkeys with cellulitis (189). Organisms formerly identified in the *E. agglomerans* complex have been placed in a new genus, *Pantoea*.

Eubacterium (see Liver Granulomas and Related Granulomatous Diseases below)

Flavobacterium

Flavobacterium is a dominant proteolytic bacterium in the upper respiratory tract of chickens and turkeys (47) that is rarely associated with clinical disease. It has been recovered from ducks with arthritis (33), an adult goose with salpingitis (35), chickens, a pigeon, and finch with septicemia and/or arthritis (250), and unhatched eggs and weak chicks (155). Heavy, pure cultures of *F. meningosepticum* were obtained from a 5-week-old ostrich chick that failed to grow and thrive and had airsacculitis, pneumonia, and thymic atrophy/hypoplasia (148).

Gaffkya (see *Peptostreptococcus*)

Gallibacterium (see *Actinobacillus/Gallibacterium*)

Hafnia

Hafnia are gram-negative rods in the family Enterobacteriaceae that are similar to *Salmonella. H. alvei* infrequently has been identified as a cause of septicemia in pullets and laying hens (50, 204). Infections were characterized by loss of appetite, diarrhea, opisthotonus, decreased egg production, and increased mortality. Scattered pale foci in the liver, hepatomegaly, splenomegaly, and catarrhal to hemorrhagic enteritis were seen on necropsy. Microscopically, degeneration, multifocal necrosis, and inflammation of the liver, lymphocytic depletion and necrosis of the spleen, and intestinal hyperemia, hemorrhage, and catarrhal enteritis were identified. Gram-negative bacteria were numerous within lesions, occurring frequently as intravascular emboli. Septicemia was reproduced following oral and intraperitoneal inoculation of pullets and hens. *Hafnia* can be identified by biochemical tests and a bacteriophage test. Specific diagnostic procedures are required to avoid misdiagnosis due to similarities between this organism and *Salmonella* species.

Helicobacter

A distinct group of bacteria, previously identified as *Campylobacter*-like organisms (see chapter 18), has been placed into the genus *Helicobacter* based on their phenotypic characteristics and 16s rRNA sequences (191). Helicobacters infecting poultry represent emerging pathogens that cause food-borne illness in people and possibly enteric and hepatic disease in poultry (9, 72).

Helicobacter pullorum, a species in the urease-negative, enterohepatic group of helicobacters, has been isolated from ceca of normal broilers, livers and intestines of layers with lesions characteristic of "vibrionic hepatitis," and people with gastroenteritis, bacteremia, and liver disease (45, 46, 229, 230, 242). The organism has been found infecting chickens in Europe and Australia (53, 169). It produces a distinct cytolethal-distending factor, which may be a significant virulence factor (54). Helicobacters can be cultured using procedures for isolating campylobacters; however, they are inhibited by polymyxin B, which was used in some older media formulations. A polymerase chain reaction to detect the organism has been developed (45, 98, 229). A multiplex PCR is useful for identifying and differentiating *Arcobacter, Campylobacter*, and *Helicobacter* (183). Specific identification requires a combination of phenotypic and genotypic analyses (98, 167, 191, 231).

Helicobacter canadensis, a species closely related to *H. pullorum*, infects geese and has been identified from people with diarrhea (258). Two additional *Helicobacter* species, *H. anseris* and *H. brantae,* were recently described infecting resident Canada geese in Boston. Although suspected to be possible human pathogens, they have not been implicated in human disease. Environmental contamination of parks by feces from geese infected with these organisms is potentially a public health concern. Another avian species (*H. pamatensis*) has been described from a tern (83) and other unnamed, distinct strains have been isolated from avian species (221).

Klebsiella

Klebsiella is an environmental contaminant that occasionally causes embryo mortality, yolk sac infections, and mortality in young chickens, turkeys, and ostriches (130, 136, 155, 192, 199, 212, 214, 253, 263). *Klebsiella* was found to be a contaminant of semen from Rhode Island red cockerels (15). Hygienic handling of semen, hatching eggs, and hatchery sanitation are necessary to prevent these losses (15, 214).

The organism has been associated with cutaneous, respiratory, ocular, systemic, and reproductive diseases of poultry. *Klebsiella* were among aerobic bacteria isolated from turkeys with cellulitis (102). Concurrent infection of young turkeys with *K. pneumoniae* increased the severity of respiratory disease resulting from *Bordetella avium* and *Chlamydiophila psittaci* infections (114). *Klebsiella* was isolated from turkey flocks with adenoviral inclusion body tracheitis that experienced respiratory disease and increased mortality. An outbreak of ocular disease caused by *Klebsiella* affected a flock of 4-week-old chickens (157). *Klebsiella* and *Staphylococcus aureus* were isolated from a septicemic disease of 20-week-old layers experiencing increased mortality. Mortality and clinical disease occurred following oral inoculation of young chicks with three *Klebsiella* biotypes. Chicks inoculated with *K. pneumoniae* had the highest mortality (81). *Klebsiella* has been infrequently isolated from reproductive diseases including salpingitis and oophoritis in hens (27, 224). Localized and systemic infections with *Klebsiella* occur in young ostriches causing "ostrich fading chick syndrome," an often-fatal disease of birds less than 3 weeks of age (243). Hydroponically grown alfalfa sprouts being fed to the birds were heavily contaminated with the organism and believed to be the source of infection (263).

Antimicrobial testing of 100 isolates from diseased or dead turkey poults between one and 35 days of age indicated sensitivity (MIC_{50} <1μg/ml) to enrofloxacin, ceftiofur, gentamicin, and trimethoprim/sufadiazine (212). Isolates (n = 22) from culled or moribund broiler chicks up to 2 weeks of age were most susceptible to ceftiofur and ciprofloxacin (136). Greater than 80% of isolates from ostriches were sensitive (MIC_{90}) to amikacin, ciprofloxacin, enrofloxacin, gentamicin, and trimethoprim/sulfamethoxazole (263).

Lactococcus

Lactococcus lactis was isolated from lungs, liver, and spleen of 5 diseased birds from over 3000 waterfowl that died in southwestern Spain. Although these were free-living waterfowl, infections in domestic waterfowl would be likely. *Lactococcus* needs to be distinguished from *Streptococcus* and *Enterococcus* (106).

Lawsonia

Lawsonia intracellularis is an obligate, intracellular, *Campylobacter*-like organism that causes proliferative enteropathy in a variety of animals, especially pigs, horses, and hamsters (147). Among avian species the disease has been reported in young ostriches and emus (66, 151). Infection of ratites was associated with increased mortality, poor growth, diarrheal disease, tenesmus, and rectal prolapse. Affected intestinal mucosa was thickened and rugose. Enterocyte proliferation, crypt changes, and infiltration of the mucosa with mixed inflammatory cells were seen microscopically. Intraepithelial, comma-shaped bacteria were visible with Warthin-Starry silver staining in the apices of enterocytes, which were identified as *L. intracellularis* by specific immunofluorescence. Affected birds responded to a 10-day course of chlortetracycline (50 mg/kg body wt) (151). Chickens were not susceptible to experimental *Lawsonia* exposure (62). Furthermore, the organism was not found in normal chickens or ones with malabsorption syndrome in the southeastern US (166). Genomic analysis of organisms from several animal species including ostriches showed they were closely related (65).

Listeria

Outbreaks of listeriosis caused by *L. monocytogenes* occur sporadically in chickens, turkeys, waterfowl, pigeons, and other avian species (107, 108, 139, 153). Young birds are most susceptible (26). The organism is important because of its ability to cause human infections following contact with infected birds (107) or consumption of contaminated poultry or poultry products, especially those that are precooked and "ready-to-eat" (74). Intestinal colonization of poultry and presence of *L. monocytogenes* in feces represent potential sources of the organism for listeriosis in ruminants (84).

Septicemic and encephalitic forms of listeriosis are recognized in birds. Emaciation and diarrhea characterize birds with septicemia. Neurological signs including depression, incoordination, ataxia, torticollis, and opisthotonos are seen in the encephalitic form (67, 69). Torticollis is especially common in affected birds. In the septicemic form, there is splenomegaly, multifocal hepatic necrosis, myocardial necrosis, and pericarditis. Myocardial degeneration, necrosis, and inflammation are often extensive (153, 200). Ascites and petechial hemorrhages in liver, heart, spleen, kidneys, and brain were seen in affected broilers (256). Salpingitis developed in hens following the acute systemic phase of the infection (135).

Birds with the encephalitic form may have visible inflammatory foci in the brain stem but usually lack gross lesions (67, 69, 107, 139, 256). Microscopically, gliosis and satellitosis in the cerebellum and hemorrhages, fibrin thrombi, and abscesses containing gram-positive bacteria are present in the midbrain, cerebellum, and medulla oblongata of birds with encephalitic listeriosis. Lesions tend to be most severe in the medulla oblongata (67, 139).

The organism is commonly found in feces and soil in temperate areas of the world. Infection can follow inhalation, ingestion, or wound contamination. An outbreak of listeriosis occurred in broilers shortly after beak trimming (256). Cold, wet conditions causing excessively moist litter were associated with an outbreak of encephalitic listeriosis and the organism was isolated from litter, water, and soil samples (67). In another outbreak, the poultry house had been flooded 10 days before onset of the disease, and conditions were hot and humid (139).

Listeria can be readily isolated and does not require special procedures (68, 181) except it may be difficult to recover from birds with the encephalitic form of the disease. However, direct culture of brain stem was positive in four of five samples collected in an outbreak of encephalitic listeriosis (69). Chicken embryos are readily infected and can be used for isolation. *L. monocytogenes* is the only species that has been implicated in poultry disease and it needs to be differentiated from other species of *Listeria* (68). There are 16 serotypes; most human and animal infections are cause by serotypes 1 and 4 (68). Demonstrating antigen in fixed tissues that have lesions of a septicemic disease is useful for confirming a diagnosis of listeriosis when culture is not possible (200). A comprehensive review of diagnostic methods for identifying *Listeria* has been published (94).

Chickens (26) and turkeys (39) are relatively resistant to experimental infection. Inoculation of younger birds and exposure to high numbers of organisms are more likely to result in colonization following oral challenge (14). Experimental air sac inoculation of day-old turkey poults with *L. monocytogenes* resulted in dose-related levels of mortality and joint infections demonstrating the feasibility of aerogenous infection. Lesions in birds that died were consistent with those that have been reported for the septicemic form of the disease (124). The organism was not shed in the eggs of heavily inoculated laying hens (164). *L. monocytogenes* has been used to study macrophage function in retrovirus infection (75) and cell-mediated responses in susceptible and resistant chickens exposed to Marek's disease virus (49).

Prevention of listeriosis depends on identifying and eliminating sources of infection. Based on the history of published outbreaks, avoiding wet conditions would seem prudent even though the risk for developing listeriosis has not been proved. The organism is often resistant to most commonly used antibiotics. High levels of tetracyclines are usually recommended for treatment. Widespread use of antibiotics in feed may have had prophylactic value in listeriosis prevention in poultry (108).

Long-Segmented Filamentous Organisms (LSFOs)

LSFOs are gram-positive, anaerobic, spore-forming bacteria that are commonly found in the jejunum and ileum of poultry and a

number of other animals. Microscopically, they can be seen embedded in the apical cytoplasm of enterocytes, displacing microvilli (268). In turkeys, LSFOs are 0.6 to 1.1μm wide and up to 13.5μm long (6). Chickens are refractory to infection with LSFOs isolated from mice even following corticosteroid treatment, suggesting that there are different types or species of LSFOs and rodents are not a likely source of infection for poultry (5). However, analysis of 16S rRNA from LSFOs infecting rats, mice, and chickens indicated they were similar and distantly related to clostridia. A provisional name, *Candidatus arthromitus,* has been proposed (225).

Often LSFOs are markedly increased in young chickens, turkeys, and quail with gastrointestinal diseases, especially during cold periods (103). While most frequently found in ill birds, LSFOs may not be pathogens, but rather they overgrow when conditions are altered because of enteric disease. The organisms stimulate mucosal immunity following phagocytosis and processing by infected enterocytes (268). In mice they provide protection against pathogen colonization and increase small intestinal transit time (226). High numbers of LSFOs were present in the jejunum of poults with experimental stunting syndrome (6), but subsequent studies using filtered inocula showed they were not the cause of the disease (218). However, depressed growth (11–14%) occurred when poults were inoculated with two isolates of filamentous bacteria (178). Presence of LSFOs has been associated with decreased carotenoid levels and skin pigmentation in chickens (4). Virginiamycin is partially effective in controlling the organisms and improving serum carotenoid levels (3).

Moraxella

Moraxella has been occasionally isolated from turkeys with respiratory disease (91). *M. osloensis* caused a cholera-like disease in commercial turkeys. Affected birds had at least one consolidated, pneumonic lung, multiple hemorrhages and inflammation of serous membranes, and abnormal spleens and livers. The organism could be distinguished from *Pasteurella multocida* by its growth on eosin-methylene blue and MacConkey agars. The disease was reproduced in experimentally inoculated turkeys (88). *Moraxella* sp. also has been recovered from salpingitis in layers (34) and from dead-in-shell embryos or weak chicks (155). An ostrich developed granulomatous conjunctivitis from which *M. phenylpyruvica* was isolated (109).

Mycobacterium avium subsp. *paratuberculosis*

Natural infections of poultry with *Mycobacterium avium* subsp. *paratuberculosis* have not been reported, but chickens are susceptible to experimental infection (247, 249) and develop an immune response following exposure to the organism (60). A related mycobacterial strain that causes a chronic intestinal disease of wood pigeons (*Columba palumbus*) in Europe (245, 248) produces lesions consistent with paratuberculosis in experimentally inoculated calves (63, 244). Both the wood pigeon strain and *M. avium* subsp. *paratuberculosis* may have significant public health importance, as they have been associated with Crohn's disease and sarcoidosis in people (165).

Neisseria

Diplococci consistent with *Neisseria* can cause pneumonia in young ostriches (122). Gram-negative, nonfermentative, *Neisseria*-like bacteria, that have a phenotype distinct from similar bacteria in the respiratory tract of chickens and turkeys, have been isolated from tracheas and lungs of birds in flocks with respiratory disease. The organism does not grow on MacConkey agar and is nonhemolytic, oxidase-positive, and catalase-positive. Turkeys were more frequently affected than chickens. Ages of infected birds ranged from 5 weeks to 3 years. Usually other bacteria or viruses were identified in affected flocks. Its role in respiratory disease is unknown (57). *Neisseria* are also commonly identified in goose venereal disease (see below).

Nocardia

Nocardia are branching, gram-positive filamentous bacteria that typically cause granulomatous lesions, especially in the respiratory system. The organism has rarely been isolated from poultry even though chickens are susceptible to experimental infection following oral or intraperitoneal inoculation (188).

Oerskovia

Oerskovia are similar to *Nocardia* and infrequently cause opportunistic infections in people and animals. The organism was isolated, along with α-hemolytic staphylococcus, from a clinically ill pigeon that had a large granulomatous mass at the base of the heart adjacent to the esophagus and trachea. Colonies of gram-positive bacilli were present within granulomas (265).

Pantoea (see *Enterobacter/Pantoea*)

Pelistega

Pelistega europaea is a newly described bacterium associated with respiratory disease in pigeons. Taxonomically it is most closely related to *Taylorella equigenitalis*, the cause of contagious equine metritis (251).

Peptostreptococcus

Young turkey poults with diarrhea often have high numbers of a large coccus in the liquid cecal contents that is typically arranged in tetrads (61). The bacterium has been referred to as *Gaffkya anaerobius*, but the genus *Gaffkya* is no longer valid. *G. anaerobius* has been transferred to the genus *Peptostreptococcus*. Genomically, peptostreptococci are more closely related to clostridia than streptococci (181). Gram-positive, anaerobic cocci, including *Peptostreptococcus*, account for up to 30% of normal cecal microbes (24). The role of the organism in diarrheal disease remains to be determined.

Planococcus

Planococcus is a halophilic, motile, gram-positive coccus that is unrelated to other cocci and is normally associated with marine environments. Pure cultures of *P. halophilus* were obtained from livers of 43-week-old layers with multifocal hepatic necrosis. Mortality of nearly 6% occurred in the flock during the month after onset of the disease. The isolate was resistant to most antimicrobials. However, it was susceptible to streptomycin, which was added to feed at 5 g/kg. Feed, specifically fishmeal and marine byproducts, was considered to be the most likely source of the organism. High ambient temperatures (up to 46°C) during the outbreak were thought to be a contributing factor (1).

Plesiomonas

Plesiomonas is closely related to *Aeromonas* and shares many of its characteristics. It is commonly found in fresh water environments and primarily infects aquatic birds (125) although it has been recovered from the livers of turkeys with histomoniasis (257).

Proteus

Proteus is a genus in the family Enterobacteriaceae that inhabits the lower intestinal tract. The organism is capable of penetrating the eggshell, which is facilitated by fecal contamination. Experimental inoculation of fertile eggs resulted in 100% embryonic mortality (44). Temperature influences egg penetration and survival within the egg (2).

Proteus occasionally causes embryonic death, yolk sac infections, and mortality in young chickens, turkeys, and ducks (25, 130, 155, 192, 199, 210, 212, 214, 253). Experimental inoculation with an isolate from ducklings failed to cause disease (210). *Proteus* also can be a contaminant of artificially collected semen (15).

Septicemia due to *Proteus* has occurred in quail (171, 211), pheasants infected with apathogenic avian influenza virus (241), and broilers suspected of having immunologic deficiency (201). *Proteus* has been recovered occasionally from a low percent of salpingitis and oophoritis lesions in layers (27, 34, 224) and has been associated with respiratory disease in chickens (156, 240, 269). An isolate from chickens with respiratory disease caused 50% mortality in experimentally inoculated 4-week-old chickens (156). *P. mirabilis* was isolated from the lung, trachea, and kidney of chickens experiencing respiratory signs, diarrhea, paralysis, and high mortality. The disease was reproduced with isolates of the organism (269). *Proteus* was isolated infrequently from turkeys with cellulitis (102, 189) and white leghorn pullets with necrotic dermatitis that seroconverted to reticuloendotheliosis virus (120). In waterfowl *Proteus* can occasionally produce arthritis, salpingitis, airsacculitis, septicemia (33, 35, 262), and granulomatous inflammation of salt glands (137).

Antimicrobial testing of 19 *Proteus* isolates from diseased or dead turkey poults between one and 35 days of age indicated sensitivity (MIC_{50} <1μg/ml) to enrofloxacin and ceftiofur (212).

Pseudomonas

Pseudomonas can cause localized or systemic diseases in young and growing poultry, invade fertile eggs causing death of embryos and newly hatched birds, and reduce shelf life of contaminated meat. The organisms are ubiquitous, often associated with soil, water, and humid environments. For a review of earlier literature on *P. aeruginosa* infections in domestic animals, see (158).

Pseudomonas aeruginosa is a motile, gram-negative, non-spore-forming rod measuring 1.5–3 × 0.5–0.8 μm, occurring singly or in short chains. The organism is a strict aerobe that grows readily on common bacteriologic media, usually producing a water-soluble green pigment composed of fluorescein and pyocyanin. A characteristic fruity odor can often be recognized. Detailed methods to characterize and differentiate pseudomonads have been published (181).

Pseudomonas is generally considered to be an opportunist that produces respiratory infections, sinusitis (91), keratitis and keratoconjunctivitis (138, 184), or septicemia and its sequelae when introduced into tissues of susceptible birds. Above normal mortality in young birds due to omphalitis and yolk sac infections acquired in the hatchery have been described (260). Chickens (17, 82, 140, 170, 184, 205, 260), turkeys (13, 102, 111, 126, 184), ducks (33, 137, 184, 210, 262), pheasants (118), ostriches (123, 175, 194, 263), geese (234), and a variety of pet and captive birds have been affected. Although birds of any age may be infected, young birds are most susceptible, as are severely stressed or immunodeficient birds. Concurrent infections with viruses and other bacteria, especially mycoplasmas, are common and may enhance susceptibility to *Pseudomonas* (198, 201, 234). Morbidity and mortality are usually 2–10% but can be much higher, approaching 100%.

Pseudomonas aeruginosa is the most common pseudomonad causing infections. Virulence is variable among isolates. Mortality following yolk sac inoculation of chicks ranged from 0–90% (260). Isolates examined in another study were highly virulent causing 50–100% mortality in experimentally inoculated 4-week-old chickens (156). *P. fluorescens* caused death of turkey embryos following dipping of eggs in contaminated antibiotic solution (22), and it has been associated with multi-causal respiratory disease of chickens (156) and turkeys (114). *P. stutzeri* was isolated from chickens with respiratory disease but produced only low mortality in experimentally inoculated chickens (156). Pseudomonads are capable of digesting eggshell cuticle if the humidity is high (37). Chickens are susceptible to melioidosis (*Burkholderia [Pseudomonas] pseudomallei*), but naturally occurring disease in poultry has not been described (255).

Mortality characterizes most *Pseudomonas* infections. Death usually occurs rapidly; often within 24–72 hours following infection. Clinical signs vary depending on whether infections are localized or systemic, but may include anorexia, stunting, lassitude, lameness, incoordination, ataxia, swelling of head, wattles, and sinuses, swelling of hock joints or foot pads, respiratory distress, diarrhea, and conjunctivitis (82, 111, 138, 140, 179). Torticollis, indistinguishable from fowl cholera, occurs following



23.25. Panophthalmitis with corneal perforation in an 18-day-old turkey poult. A heavy pure growth of *Pseudomonas aeruginosa* was obtained on culture, and intralesional bacteria were present in tissues on histopathology. (Tahseen Abdul-Aziz)



23.26. Subcutaneous lesions in the upper neck area of chicks following the use of a Marek's disease vaccine contaminated by *Pseudomonas*. (Laddie Munger)

Pseudomonas inoculation of turkeys via the eustachian tube (190). Infections of the nasal glands in ducks resulted in granulomatous adenitis (137).

Lesions are consistent with clinical findings and include subcutaneous edema and fibrin, occasionally with hemorrhage, exudate in affected joints, inflammation of serous membranes mimicking lesions of colisepticemia (airsacculitis, pericarditis, perihepatitis), pneumonia, swelling and necrotic foci in liver, spleen, kidney, and brain, conjunctivitis, sinusitis, and occasionally keratitis (82, 111, 138, 156, 179, 184). Chicks that survived yolk sac inoculation had retained, inflamed yolk sacs when examined 14 days postinoculation (260). Unilateral panophthalmitis characterized by corneal perforation and phacolysis occurred in young turkeys with ocular *Pseudomonas* infection (Fig. 23.25). The rapidly progressive destruction of the eye may be related to proteases produced by the organism (13). Large numbers of bacteria, often in and around affected blood vessels within most tissues, including brain, are typically seen microscopically in acute lesions. Heterophilic exudate in the pharynx and pulmonary foci were present in respiratory infections of pheasants (118). A similar case characterized by pseudodiptheretic membranes and granulomatous lesions in the respiratory and upper digestive tract occurred in a group of young ostriches experiencing high mortality. Intralesional *P. aeruginosa* colonies were identified by immunohistochemistry and the infection was confirmed by culture (175). *Pseudomonas* was isolated infrequently from adult hens with salpingitis and oophoritis (27, 224), from turkeys with cellulitis (102, 189), from geese with venereal disease (see below), and was the most common bacterium recovered from abnormal joints of broiler chickens in a study of leg weakness (40).

Pseudomonas is among a variety of bacteria often recovered from dead embryos and sick newly hatched chickens, turkeys, pheasants, ducks, and ostriches (44, 80, 130, 155, 192, 210, 253). With the exception of a respiratory outbreak in pheasants attributed to exploding contaminated eggs in the incubator, presence of *P. aeruginosa* in embryos is not considered a source of infection for older birds. Severe outbreaks have followed injection of large numbers of birds with contaminated vaccines (Fig. 23.26) (170, 246) and antibiotic solutions (52, 266). In these cases, contamination resulted from poor hygiene during mixing and handling, not from the products themselves. Contact with infected birds (179) and intense, continuous broiler production with different ages being raised at the same facility (82) can result in spread of *Pseudomonas* infection. In some outbreaks, the source of the organism and how it spread could not be determined.

Diagnosis requires isolation and identification of the organism. Various methods including serologic, phage, and aeruginocine typing methods are available (209) and may be useful in epidemiologic studies.

Prevention and control are based on identifying and eliminating the source of the organism. Good hygiene, especially in hatcheries and when birds are injected, is fundamental to *Pseudomonas* control. Cleaning and disinfection of equipment and use of sterile techniques in preparing vaccines and injectables will control *Pseudomonas* infections resulting from inoculation (52). Sensitivity of isolates to hatchery disinfectants needs to be determined (267). A commercial quaternary ammonium disinfectant was ineffective at achieving total kill of high concentrations (10^9) of *P. aeruginosa* that had been isolated from chicks with omphalitis. It was generally effective when numbers of organisms were low (10^3) and had a variable effect on intermediate concentrations (10^6). Potentiation of the disinfectant with EDTA substantially improved its efficacy when tested *in vitro* (259). Use of either quaternary ammonium with or without EDTA reduced airborne bacteria at hatching and did not have an adverse affect on hatchability or livability (261). Reduction of stress and prevention of other viral and bacterial infections will aid in reducing susceptibility to *Pseudomonas*.

Antibiotics can be useful in reducing losses if initiated early in

the disease, but because of the organism's resistance to many antimicrobials (140, 156, 193, 195, 205, 209, 260), sensitivity testing is essential. Antimicrobial testing of 31 isolates from diseased or dead turkey poults between one and 35 days of age indicated sensitivity (MIC_{50} <1μg/ml) only to enrofloxacin (212). In contrast, greater than 80% of isolates from ostriches were sensitive (MIC_{90}) to amikacin, ciprofloxacin, enrofloxacin, and gentamicin (263). Supplemental vitamin A and potassium permanganate in the water were helpful adjuncts to antibiotic therapy in controlling conjunctivitis (138).

Rothia

Rothia species are aerobic actinomycetes that have been associated with chronic infections, most notably tooth decay, in human beings and animals. They are closely related to *Actinomyces. Rothia* was the only bacterium isolated from osteomyelitis and joint lesions in four lame or recumbent tom turkeys in an affected flock. Intravenous inoculation of unaffected turkeys reproduced the clinical signs and lesions from which the organism was reisolated (22).

Streptobacillus

Streptobacillus moniliformis, a gram-negative, often beaded, nonbranching, filamentous bacterium, can infect turkeys, usually following rat bites or exposure to infected rats. Polyarthritis and synovitis occur in infected birds; other tissues are usually normal. The disease can be reproduced in turkeys following experimental inoculation of the organism by intravenous, subcutaneous, and footpad routes, but not by oral administration. Chickens are not susceptible. Diagnosis requires isolation and identification of the organism. Infection can be prevented through rodent control (100, 172).

Streptomyces

Normally a free-living soil organism, *Streptomyces* was isolated in pure culture from nonviable ratite eggs. Large gray-white plaques were present between the eggshell and shell membrane and the dead embryos were congested, had abnormal discolored yolk, and had multiple white foci in the liver. Washing and disinfecting of the egg was believed to have contributed to the infection (263).

Suttonella

Unique gram-negative rods isolated from passerine birds experiencing episodes of mortality in Great Britain were described as a new species, *Suttonella ornithocola* (92, 134). The organism has not been associated with disease in poultry.

Vibrio

Non-O1 *V. cholerae* has been isolated from geese that died following weight loss and lassitude of 2–3 days duration (216), from nasal cavities of apparently healthy ducks (32), and from tissues of ducks with airsacculitis or septicemia (262). Individuals working with ill birds that have contact with coastal waters and shellfish need to be aware that birds can be a source of human infection (216). Conjunctivitis caused by *V. cholerae* NAG, a potential human pathogen, occurred in ducklings (31). The organism was also isolated from the intestines, and water where the ducks were being kept. Exposure of the domesticated ducks was believed to have come from free-living birds. Both O1 and non-O1 *V. cholerae* have been frequently isolated from feces of aquatic birds and their environments (187).

Historically, a cholera-like disease has been reported in poultry and zoo birds caused by *V. metschnikovii (metschnicovii)*, and campylobacters have been implicated as the cause of hepatitis in chickens (see Chapter 18) (196). *V. metschnikovii* is still occasionally isolated from diseased chickens (149) and waterfowl (115). An outbreak of vibrionic hepatitis in a layer flock caused an 89% decrease in egg production and 10% increase in mortality. Lesions included hydropericardium, ascites, and an enlarged, discolored liver that contained multiple gray to yellow necrotic foci. Chlortetracycline was effective in controlling the disease (215).

Vibrio alginolyticus is a dominant proteolytic bacterium in the upper respiratory tract of chickens and turkeys. It does not cause disease but may enhance pathogenicity of avian influenza viruses by providing a mechanism for cleavage of the virus hemagglutinin (47).

Diseases Caused by or Associated with Bacteria

Beak Necrosis

A gram-positive bacterium with affinity for keratin was associated with beak necrosis that affected nearly half of the birds in a flock of 1-year-old broiler breeder hens and caused approximately 10% mortality (56). Feeding fine feed (mash) predisposes birds to oral and beak lesions although the exact mechanism of how lesions develop is unknown. Oral lesions resolve rapidly after birds are put on pelleted feed (95). Injury to the epidermis occurs initially followed by necrosis, ulceration, and bacterial growth. Affected birds have decreased leukocytes and anemia (96). Beak deformity, loss of the distal end of the mandible, and osteomyelitis occur in severely affected birds. Weight loss and mortality result from impaired feeding (86). Use of 40-mm grids over feeders to restrict male access to feed resulted in a higher occurrence of oral and beak lesions (116). Males are more often affected than females (86). Visibly affected birds need to be culled for welfare reasons. In one study, beak lesions were the major cause for culling of male broiler breeders (117).

Goose Venereal Disease

An infectious venereal disease of uncertain etiology characterized by inflammation of the penis and cloaca of ganders in breeding flocks was first described in Hungary (239). Subsequently, the disease has affected flocks in Europe, Russia, and the Middle East. Initially, the base of the phallus becomes

swollen and inflamed, with the process extending to the cloaca. Later, there is necrosis, ulceration, and eventually considerable scarring of the mucosa, often making reproduction impossible. Similar lesions may develop in the cloaca of hens following breeding. Morbidity ranges from 20 to 100%, and newly introduced birds readily contract the disease. Increased infertility and gander mortality of approximately 5% are flock problems resulting from the disease (232).

A variety of bacteria, especially *Neisseria* and *Mycoplasma* spp. and *Candida albicans*, affecting the phallus of ganders and cloaca of hens has been associated with the disease (29, 162, 232, 233). The normal phallus microflora of unaffected ganders has been established (161) and is similar, with the exception of *Mycoplasma* and *Candida albicans*, to that of affected ganders (29). Use of antimicrobials effective against mycoplasmas substantially reduces the severity of the disease (77). A similar disease attributed to *C. albicans* spread through goose flocks in Israel (28). Dramatic improvement followed treatment with mycostatin and antibiotics. Vaccination with ethanol-inactivated fungus provided good control of the disease (141). A phycomycete, *Mucor janssenii*, found in one flock of affected ganders reproduced a similar disease in experimentally inoculated birds (163). Exposure of SPF Muscovy ducks and geese to isolates alone and in various combinations produced only mild clinical signs and lesions except in a female contaminated by an affected gander and inoculated with *C. albicans*. Trauma is considered to be a likely initiating factor followed by infection with bacteria and fungi (162).

It is recommended that ganders be examined at each breeding season and affected birds removed from the flock. Artificial insemination can be used in affected flocks to improve fertility (29).

Intracellular Infection in Ducks

Mortality in Muscovy ducks (*Cairina moschata*) caused by an intracellular organism primarily affecting endothelial cells in the lungs was initially attributed to *Haemoproteus* infection (127). However, subsequent examination of additional cases revealed that the organism was not a protozoan, but probably a bacterium capable of forming spores or an unidentified microorganism. Muscovy ducks are most susceptible and can contract the infection from asymptomatic infected Pekin ducks. Experimental transmission is possible using blood from an infected duck.

Lungs of affected ducks are dark red-purple, slightly edematous, and firm. Microscopically, air capillaries are obliterated because of marked swelling of endothelial cells, which are often packed with intracellular organisms, and interlobular septa are widened and contain inflammatory cells and edema. In tissue sections, organisms stain poorly with hematoxylin and eosin but are readily demonstrated with periodic-acid-Schiff or silver stains (128, 202).

Liver Granulomas and Related Granulomatous Disorders

Granulomas are occasionally seen in livers of turkeys and, less frequently, chickens at processing. Affected livers and carcasses are condemned. The incidence in individual flocks may reach 50%.

Granulomas are focal or multifocal, single to coalescing lesions that are grossly visible as firm, lobulated, roughly spherical, pale yellow to white masses ranging in size from a few millimeters to several centimeters. Advanced lesions have a rough appearance and may be "gritty" when cut. Bile stasis of adjacent normal hepatic tissue is often marked. Liver granulomas are caused by a variety of infectious and parasitic agents. *E. coli, Eubacterium*, and other bacteria are among the more common etiologic agents (235). Similar granulomas are occasionally seen in the spleen and rarely in other tissues of affected birds.

Microscopically, lesions are typical avian heterophilic granulomas (176) that contain a central caseous mass covered by a layer of multinucleated giant cells, which is confluent except in areas where the process is still active. Surrounding the caseated center is a more diffuse zone of heterophils, macrophages, fibroblasts, and lymphocytes. Heterophils can be seen migrating through the layer of giant cells and are especially numerous in areas where giant cells are absent or discontinuous. Diffuse and focal lymphocytic foci form the outermost layer of the lesion. Fibrosis may be extensive in chronic lesions. Bacteria generally are not visible unless special stains are used. Tangles of filamentous organisms can usually be seen with silver stains such as Warthin-Starry or Dieterle and gram-positive filamentous or coccoid organisms may be seen with Gram's stain (8, 146, 177).

A variety of bacteria have been isolated from the lesions including *Actinomyces* (219), *Catenabacterium, Corynebacterium, Eubacterium, Propionibacterium, Enterococcus (Streptococcus)*, and *Staphylococcus* (146, 177). Liver granulomas occurred after intravenous inoculation of turkeys with a *Catenabacterium* spp. that had been isolated from a naturally infected turkey. Chickens, peafowl, guinea pigs, rabbits, hamsters, and mice did not develop lesions (177). Granulomatous lesions in liver and spleen were reproduced experimentally by inoculating turkeys with *Eubacterium tortuosum* (8, 112) even though the organism is part of the normal cecal flora (112). Co-inoculation with other bacteria, including a virulent strain of *E. faecalis* or *S. epidermidis*, increased the likelihood of liver lesions. Often, mucosal ulcers in the lower intestinal tract can be found in affected birds suggesting liver lesions develop from bacteria carried to the liver from the intestine via the bloodstream (8, 112, 146, 177).

Liver granulomas also occur in chickens but much less frequently. Gram-positive, filamentous bacteria morphologically and tinctorially distinct from *Eubacterium*, long-segmented filamentous organisms, *Actinomyces*, and *Nocardia* were present in sporadic cases of visceral granulomas in broiler chickens at processing in the United States (113). Lesions also occurred in the spleen, cecum, and mesentery of some birds.

In 7–8-week-old turkeys, pyogranulomatous typhlitis and hepatitis characterized by cecal cores and rupture was associated with *E. coli,* and concurrent coccidia and hemorrhagic enteritis virus infections. Excess condemnations for granulomatous lesions did not occur when the flock was processed (178).

Larvae of intestinal helminths also can cause hepatic foci ("white-spotted livers") in turkeys and chickens that need to be differentiated from bacterial granulomas. Only a low percentage of hepatic foci in turkey livers at processing yielded bacteria;

E. coli and *Salmonella* spp. were most commonly recovered (185). Foci were associated with migration of *Ascaridia* larvae. Exposing turkey poults to *A. dissimilis* ova reproduced the lesions (186). No causative organism initially was identified in granulomatous lesions in ceca and livers of older chickens from small flocks in Canada (182), but subsequently, larvae of the cecal worm, *Heterakis gallinarum*, were found to be associated with the lesions (206).

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Chapter 24

Avian Chlamydiosis (Psittacosis, Ornithosis)

Arthur A. Andersen and Daisy Vanrompay

Introduction

Definition and Synonyms

Avian chlamydiosis is caused by the bacterium *Chlamydophila psittaci*. Avian chlamydiosis in birds is usually systemic and occasionally fatal. Clinical signs vary greatly in severity and depend on the species and age of the bird and the strain of chlamydia. Avian chlamydiosis can produce lethargy, hyperthermia, abnormal excretions, nasal and ocular discharges, and reduced egg production. Mortality ranges up to 30%. In pet birds, the most frequent clinical signs are anorexia and weight loss, diarrhea, yellowish droppings, sinusitis, and respiratory distress (64). Many birds, especially older psittacine birds, may show no clinical signs; nevertheless, they will often shed chlamydiae for extended periods. Necropsy of infected birds often will reveal spleen and liver enlargement, fibrinous air sacculitis, pericarditis, and peritonitis (72, 89, 101).

This chapter primarily covers avian chlamydiosis as it occurs in birds raised commercially for meat and egg production—turkeys, ducks, and pigeons. It should be noted that the disease in pet birds is quite similar, and the disease characteristics, transmission, and diagnosis are essentially the same. A summary of the disease and control procedures for chlamydiosis in companion birds was recently published (84) and is on the American Veterinary Medical Association (AVMA) and Centers for Disease Control and Prevention (CDC) Web sites.

The disease in birds and humans originally was called psittacosis or parrot fever (65) as it was first recognized in psittacine birds and in humans associated with psittacine birds. Ornithosis was a term introduced in 1941 by Meyer (63) to differentiate the disease in or contracted from domestic and wild fowl from the disease in or contracted from psittacine birds. The two syndromes are currently considered to be the same (69). Their earlier separation was based on the assumption that in man ornithosis was a milder disease than psittacosis. However, the disease in man contracted from turkeys often is more severe than that contracted from psittacine birds.

Public Health Significance

Avian strains of *C. psittaci* can infect humans, and precautions should be taken when handling infected birds or contaminated materials. Human infections are common following handling or processing of infected turkeys or ducks. Most infections are through inhalation of infectious aerosols; thus, processing plant employees are especially at risk, as are farm workers and poultry inspectors at processing plants. Personnel who are employed to further process turkey meat have also become infected. Chickens, pigeons, pheasants, quail, and partridges also may pose public health threats, mainly to their producers.

In humans, the incubation period of avian chlamydiosis is usually 5–14 days; however, longer periods are known (84). Infections vary from inapparent to severe systemic disease with pneumonia. Because the disease is rarely fatal in properly treated patients, awareness of the danger and early diagnosis are important. Infected humans typically develop headache, chills, malaise, and myalgia, with or without signs of respiratory involvement. Although pulmonary involvement is common, auscultatory findings may appear normal or underestimate the extent of involvement. A human chlamydial strain (*C. pneumonia* strain TWAR) produces similar disease symptoms in humans (33, 34). The recommended therapy for it is the same as for the avian strain, so a differential diagnosis is not required.

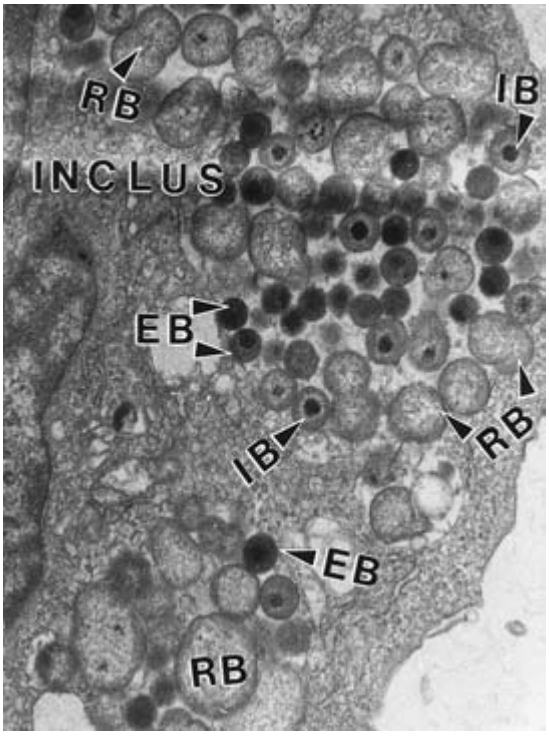
History

Avian chlamydiosis gained world prominence during a pandemic in 1929–1930 that involved at least 12 countries. In the United States, the disease was attributed to the importation of green Amazon parrots from South America. In 1931, strict regulations were placed on the importation of parrots from tropical countries. During this time, Leventhal, Cole, and Lillie independently observed very small basophilic bodies in the tissues of infected birds and humans and suggested that they were the causative agent. The etiological relationship between basophilic bodies and disease was soon established conclusively by Bedson and Bland (cited in 61).

During the next 20 years, it became clear that chlamydiae were not limited to psittacine birds, were widespread in almost all avian species, and that chlamydiae from other avian species were transmissible to humans. In 1939, chlamydiae were isolated from two pigeons sent to the diagnostic laboratory in South Africa by a pigeon fancier who was losing a few birds from his flocks. Isolates were soon recovered from racing and carrier pigeons in California, and infections in two humans in New York were attributed to contact with feral pigeons. In 1942, serological evidence showed that ducks and turkeys could be infected naturally. Within 3 years, human infections due to contact with ducks were reported in California and New York. However, it was not until the early 1950s that isolates were made both from turkeys and from humans in contact with the turkeys (61). The list of avian

species in which naturally occurring chlamydial infections were identified increased rapidly until today more than 400 species of birds belonging to more than 21 orders have been reported.

During the 1960s, the incidence of severe epidemics in poultry in the United States and Europe declined, although occasional outbreaks and serologic evidence show that avian chlamydiaosis is a continuing threat both to birds and to humans in contact with them. Outbreaks in turkeys were reported in the United States during the 1980s (40, 67) and more recently in Europe (81, 104). An increase in the number of outbreaks due to chlamydia in ducks has been reported in recent years. Human infections were associated with a number of these outbreaks (11, 15, 32, 50, 57, 59, 66).



24.1. Transmission electron photomicrograph of a *C. psittaci* inclusion (INCLUS) in infected L929 cells. The various morphological forms of chlamydiae are present: elementary body (EB), reticulate body (RB), and intermediate body (IB). ×15,000

Etiology

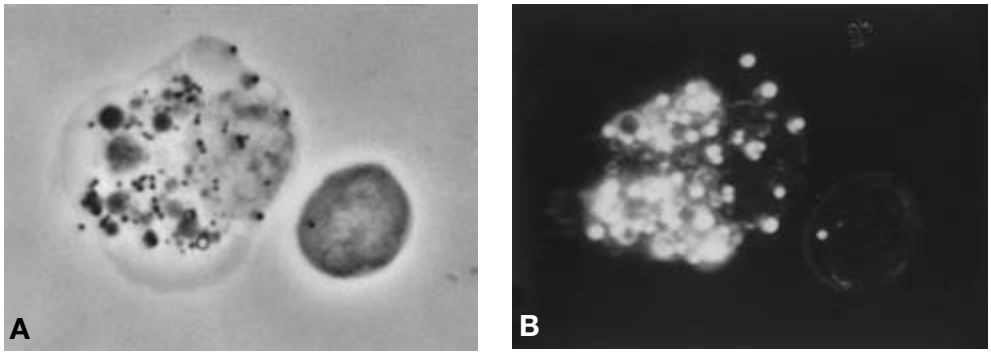
Classification

The specific genus and species will be used when referring to species and strains. The members of the order Chlamydiales are obligate, intracellular gram-negative bacteria infecting both humans and animals. Rapid and easy identification of these bacteria is essential for taxonomic, epidemiological, and clinical determinations. The family Chlamydiaceae was recently reclassified into two genera, *Chlamydia* and *Chlamydophila* (25). The two genera, *Chlamydia* and *Chlamydophila,* correspond to the former *C. trachomatis* and *C. psittaci*, respectively. Under the new classification, *Chlamydia* includes 3 species (*C. trachomatis, C. muridarum,* and *C. suis*), and *Chlamydophila* includes 6 species (*C. psittaci, C. abortus, C. felis, C. caviae, C. pneumoniae,* and *C. pecorum*). DNA sequence analysis of phylogenetic markers (the ribosomal intergenic spacer and the domain I of the 23S rRNA gene) provides a rapid and reproducible method for identifying, grouping, and classifying chlamydial strains (25, 27, 80). For continuity with the old classification, the terms chlamydiosis, chlamydiae, etc., are used as generic terms and refer to the diseases caused by all members of the genera *Chlamydia* and *Chlamydophila.*

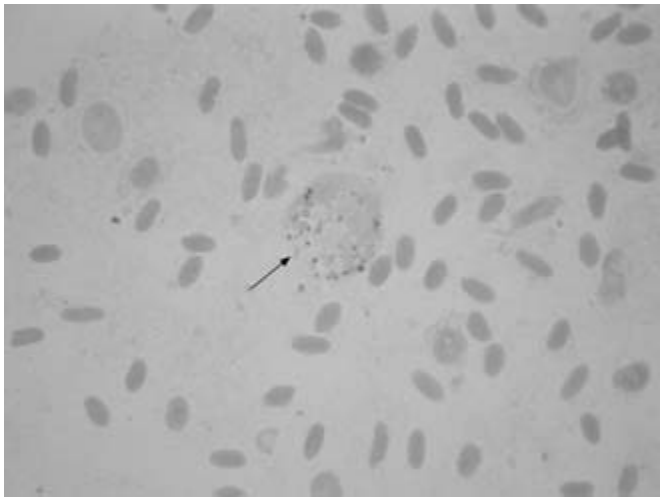
Morphology and Staining

The three morphologically distinct forms of *Chlamydophila* are termed elementary body (EB), reticulate body (RB), and intermediate body (IB) (Fig. 24.1). The EB is a small, electron-dense, spherical body, about 0.2–0.3 mm in diameter, which rivals mycoplasma for the smallest of the prokaryotes. The EB is the infectious form of the organism, which attaches to the target epithelial cell and gains entry. The EBs are characterized by a highly electron-dense nucleoid, located at the periphery of the EB and clearly separated from an electron-dense cytoplasm. Following entry into the host cell, the EB expands in size to form the RB, which is the intracellular metabolically active form. It is larger than the EB, measuring approximately 0.5–2.0 mm in diameter. The RB divides by binary fission and thereafter matures into new EBs. During this maturation, morphologically intermediate forms (IB), measuring about 0.3–1.0 mm in diameter, can be observed inside the host cell. The IB has a central electron-dense core with radially arranged individual nucleoid fibers surrounding the core. Cytoplasmic granules are tightly packed at the periphery of the IB and are separated from the core by a translucent zone.

All chlamydiae are gram-negative, but the Gram stain is of no practical value in identifying chlamydiae. Chlamydiae are large enough to be seen either with a light microscope using special optics or with selective stains. In wet mounts of impression smears of infected tissues or exudates, intracellular chlamydiae are large enough to be seen at magnifications of 3800 or more in phase contrast microscopes (Fig. 24.2A). They are also readily seen by dark-field illumination (Fig. 24.2B). With either technique, however, they cannot be distinguished from contaminating intracellular mycoplasma organisms. When only bright-field optics are available, chlamydiae may be seen in touch impressions of infected tissues by staining them with Castaneda, Giemsa, Gimenez, Machiavello, or Stamp methods after appropriate fixation (2). They appear dark purple with Giemsa; blue with Castaneda; and red with Machiavello, Gimenez, and Stamp stains against contrasting backgrounds. The Gimenez method (31) is preferred for staining chlamydiae in touch impressions of yolk sacs of infected chicken embryos and has proved very useful in obtaining presumptive diagnoses in microscopic examination of touch impressions of diseased air sacs, spleens, and pericardia of naturally infected birds (Fig. 24.3).



24.2. A. Phase contrast photomicrograph of chlamydia-laden mononuclear cell in air sac exudate of turkey infected with *C. psittaci*. B. Dark-field photomicrograph of mononuclear cell in A (70). ×4000



24.3. Gimenez staining of a pigeon's spleen. Notice the macrophage (arrow) with the purple-red colored chlamydial inclusions. Light-microscopic photomicrograph.

Biochemical Properties

The RB is the intracellular, metabolically active form. Although DNA and RNA are both found in the EB and the RB, the ratio of RNA to DNA is greater in the RB. The RBs synthesize their own DNA, RNA, and protein, but some of their metabolic capabilities are limited when compared with free-living, colonizing bacteria. For example, they cannot complete the pentose cycle and do not use pyruvate by way of the tricarboxylic acid cycle. They can, however, catabolize pyruvic, aspartic, and glutamic acids, generating CO_2 and 2- and 4-carbon residues.

Antibiotic Susceptibility

Multiplication of all strains of chlamydiae (except for some *Chlamydia suis* strains and experimental mutants) is strongly inhibited by appropriate concentrations of tetracyclines, chloramphenicol, and erythromycin and less so by penicillin. Some strains are inhibited by D-cycloserine. All strains of *C. trachomatis* are inhibited by sodium sulfadiazine. By varied mechanisms tetracyclines, chloramphenicol, and erythromycin inhibit synthesis of protein on chlamydial ribosomes. Penicillin interferes with chlamydial cell wall synthesis, resulting in the interruption of RB binary fission and, thus, the formation of abnormally large RBs that cannot mature into EBs. D-cycloserine acts similarly, but the drug's action can be reversed by the addition of alanine. Inhibition of multiplication by sodium sulfadiazine affects the organism's ability to produce folic acid; this inhibition can be reversed by the addition of *p*-aminobenzoic acid. Certain antibiotics have little or no effect on the growth of chlamydiae, and this fact can be useful in selecting for viable chlamydiae in suspensions containing contaminating bacteria. Concentrations of 1 mg/ml of streptomycin sulfate, vancomycin, and kanamycin sulfate may be used for this purpose. Chlamydiae are also unaffected by bacitracin, gentamicin, and neomycin.

Susceptibility to Chemical and Physical Agents

Chlamydiae are highly susceptible to chemicals that affect their lipid content or the integrity of their cell walls. Even in a milieu of tissue debris, they are inactivated rapidly by surface-active compounds, such as quaternary ammonium compounds and lipid solvents (84). They are somewhat less susceptible to dilute solutions of protein denaturants, acids, and alkalies (methanol, ethanol, ammonium or zinc sulfate, phenol, hydrochloric acid, or sodium hydroxide). Infectivity is destroyed within minutes, however, by exposure to common disinfectants such as benzalkonium chloride, alcoholic iodine solution, 70% ethanol, 3% hydrogen peroxide, and silver nitrate; but they are resistant to cresol compounds and lime. Dilute suspensions (20%) of infectious tissue homogenates are inactivated by incubation for 5 minutes at 56°C, 48 hours at 37°C, 12 days at 22°C, and 50 days at 4°C (73).

Infectious dense forms of the organisms in yolk sac membranes or mouse tissues may be preserved indefinitely at –20°C or below, although the initial freezing and subsequent thawing incurs a titer loss of 1–2 $\log_{10}$. Infectivity of the suspension is destroyed after 6 freeze-thaw cycles (73). Thin-walled, large forms of the organism are inactivated at –70°C.

Cell walls of the dense forms are disrupted by ultrasonification at frequencies above 100 KC or by treatment of intact organisms with sodium deoxycholate.

Antigenic Structure and Toxins

The number of proteins produced by chlamydiae is unknown, and only a limited number have been studied for their antigenic importance. The cystine-rich major outer membrane protein

(MOMP) has a molecular weight of 40 kDa and represents approximately 60% of the weight of the outer membrane. The MOMP is an immunodominant protein, and there is considerable evidence that antibodies to surface-accessible epitopes of MOMP have a protective role in immunity to chlamydial infection. The outer membrane protein A (*ompA*) gene (also referred to as *omp1* gene) encodes the MOMP. The *ompA* gene contains 5 conserved- and four variable-sequence regions, VS1 to VS4, which encode for the variable-protein domains VDI to VDIV. VDI, VDII, and VDIV especially protrude from the *C. psittaci* membrane. Epitope mapping has shown the presence of genus- and species-specific antigenic determinants within the conserved regions. However, species-specific antigenic determinants have also been found in the most conserved parts of VDIV. Serovar-specific antigenic determinants are located within VDI and VDII. Monoclonal antibodies (MAbs) to the highly immunoaccessible serovar-specific epitopes on the MOMP can passively neutralize chlamydial pathogenicity and infectivity. Monoclonal antibodies to genus-, species-, or serovar-specific epitopes on the MOMP are excellent tools for specific chlamydial diagnosis.

The chlamydial lipopolysaccharide (LPS) is also an essential constituent of the outer membrane and, like the MOMP, represents one of the major surface-exposed antigens of chlamydiae in both the EB and the RB. It has a molecular weight of 10 kDa and is chemically and serologically related to the LPS of gram-negative enterobacteriaceae. In fact, the chlamydial LPS contains several antigenic determinants cross-reacting with the LPS of enterobacterial Re mutants of *Salmonella* species and *Acinetobacter calcoaceticus* (17, 68). However, the chlamydial LPS contains in its saccharide moiety, a trisaccharide of 3-deoxy-D-manno-2-octulosonic acid (Kdo) of the sequence αKdo (2 → 8) -αKdo- (2 → 4) -aKdo. This antigenic epitope is shared only by all members of the genera *Chlamydia* and *Chlamydophila* and, thus, represents a Chlamydiaceae-specific antigen useful for specific diagnosis (18).

A chlamydial cystine-rich heat shock protein 60 (hsp60) has been described that is cross-reactive with other gram-negative bacteria such as *Escherichia coli, Neisseria gonorrhoeae,* and *Coxiella burnetii* (107). This is not surprising considering the fact that heat shock proteins are among the most conserved molecules in phylogeny. Thus, the presence of cross-reactive epitopes on the chlamydial outer membrane should be kept in mind when choosing or interpreting a specific diagnostic test. The chlamydial hsp60 has been associated with the hypersensitivity often seen in repeated chlamydial infections. It is thought to play a major role in scar formation and the sequelae seen in *C. trachomatis* infections of the eye and reproductive tract. Specific toxins are not yet identified in chlamydiae.

Strain Classification

Antigenicity, Genetic or Molecular

Chlamydophila psittaci includes 8 known serovars (serotypes) (Table 24.1) designated A to F, M56, and WC (5, 8, 103). Of the 8 known serotypes in the species *C. psittaci*, 6 are known to naturally infect birds. These serotypes are distinct from those associated with chlamydiosis in mammals. Each avian serotype appears to be associated with a different group or order of birds (8, 103). Serovar A is endemic among psittacine birds and causes sporadic zoonotic disease in pet bird owners. Serovar B is endemic in pigeons but also has been isolated from other bird species. Serovar B strains are potentially hazardous to pigeon fanciers, although these strains seem to be less pathogenic to humans in comparison with serovar A strains. Water fowl most frequently seem to be infected with serovar C strains, although serovar C has also been isolated from a turkey (CT1) and a partridge (Par1). Serovar D is highly virulent and is often associated with turkeys but has also been isolated from egrets and seagulls. Veterinarians and poultry workers are especially at risk of becoming infected with serovar D strains. Serovar E (also known as Cal-10, MP, or MN) was first isolated during an outbreak of pneumonia in humans during the early 1930s (28). Later on, serovar E isolates were obtained from a variety of bird species including ducks, pigeons, ostriches, and rheas. Serovar F is represented by the psittacine isolate VS225. The M56 serovar was isolated during an outbreak in muskrats and hares (85). The WC serovar was isolated during an outbreak of enteritis in cattle (71).

Table 24.1. *Chlamydophila psittaci* serotypes.

Serotype	Representative chlamydial strain	Host association
A	VS1	Psittacine
B	CP3	Pigeons, doves
C	GR9	Ducks, geese
D	NJ1	Turkeys
E	MN	Pigeons, turkeys
F	VS225	Psittacine
M56	M56	Muskrat, snowshoe hare
WC	WC	Cattle

All Chlamydiaceae are recognized by monoclonal antibodies (MAbs) that detect the LPS αKdo (2 → 8) -αKdo- (2 → 4) -αKdo. A multiplex-PCR test that targets the MOMP gene, tRNA-gly, and 23S rRNA also recognizes all Chlamydiaceae (25). *Chlamydia* species have a common antigenic epitope in variable segment 4 of the MOMP: NPTI, TLNPTI, LNPTIA, or LNPTI. Monoclonal antibodies should recognize this epitope in *Chlamydia*, to the exclusion of *Chlamydophila*. However, DNA sequencing is the most reliable way to distinguish *Chlamydia* and *Chlamydophila.* Sequencing of the PCR amplified Chlamydiaceae 16S–23S rRNA intergenic spacer allows the identification of Chlamydiaceae genera (25).

Species within *Chlamydia* and *Chlamydophila* are distinguished by either serology, restriction fragment length polymorphism (RFLP), or at least partial DNA sequence analysis of any one of 8 genetic loci: MOMP, GroEL, 60 kDa cysteine-rich protein, small cysteine-rich lipoprotein, KDO-transferase, 16S rRNA, 23S rRNA, or the 16S–23S intergenic spacer (19). The 8 known *C. psittaci* serovars (A to F, M56, and WC) can be distinguished by use of a panel of serovar-specific monoclonal antibodies in a micro-immunofluorescence test (5, 8). However,

serotyping can be performed only in specialized laboratories because serovar-specific MAbs are not commercially available. Genotyping by *AluI* restriction fragment length polymorphism (RFLP) analysis of the *ompA* gene, encoding the MOMP, was introduced in 1995 (83). RFLP analysis of the *ompA* gene of all known *C. psittaci* serovars revealed corresponding restriction patterns or genotypes (5, 96). More recently, *ompA* genotyping by real-time PCR using genotype-specific probes has been described, allowing the detection of an additional variant described as genotype E/B (29). Genotyping of avian *C. psittaci* strains is very convenient, as it is a rapid, powerful technique that can be used directly on clinical samples. The method of choice will depend on the laboratory, as either analysis can be used to classify the avian *C. psittaci* isolates.

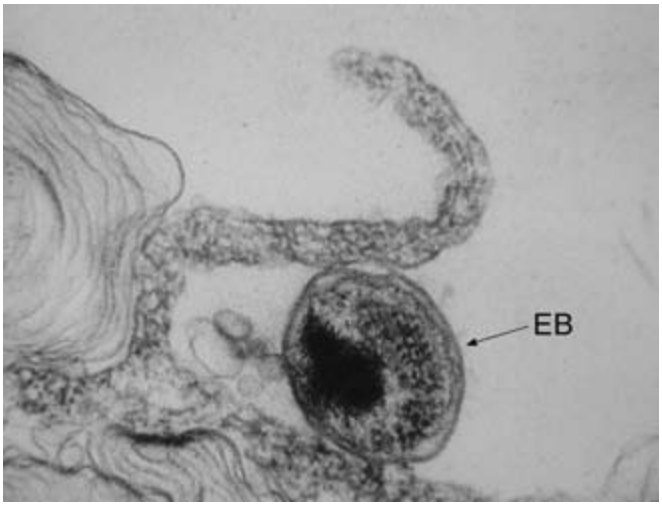
Pathogenicity

Based on natural pathogenicity for domestic fowl, strains of *C. psittaci* isolated from birds fall into two general categories: 1) Highly virulent strains that cause acute epidemics in which 5–30% of affected birds die and 2) less virulent strains that cause slowly progressive epidemics. Strains of both high and low virulence appear to have equal ability to spread rapidly through a flock, as evidenced by serologic test results. Highly virulent strains are isolated most often from turkeys and occasionally from clinically normal wild birds. The isolates serotyped from some of the earlier outbreaks with high mortality have been of serotype D (8, 105). These strains are also labeled "toxigenic," because in natural and experimental hosts they produce rapidly fatal disease with lesions characterized by extensive vascular congestion and inflammation of vital organs. Toxigenic strains have a broad spectrum of pathogenicity for laboratory animals and can cause serious human infections (some fatal) in poultry handlers and laboratory research workers. Strains of low virulence cause slowly progressive epidemics with a mortality rate of less than 5% when uncomplicated by secondary bacterial or parasitic infection. Strains of this category are routinely isolated from pigeons and ducks and occasionally from turkeys, sparrows, and other wild birds. The turkey isolates from outbreaks with low mortality have been of serotype B or E. Birds infected with these strains usually do not develop the severe vascular damage typical in birds infected with the virulent toxigenic strains, nor do they have the severe clinical signs (89).

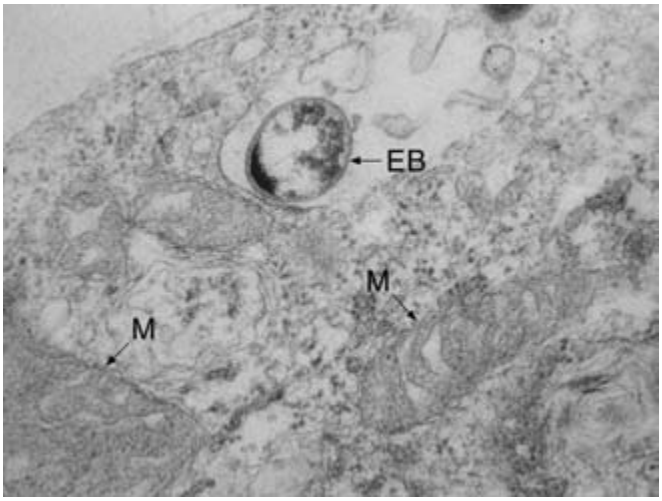
Chlamydiosis in pigeons, ducks, and some psittacine birds often is accompanied by concurrent infection with salmonellae. In such cases, the mortality rate among birds is high, and chlamydiae are shed in very large numbers; susceptible hosts in the immediate environment of the infected birds are exposed to doses that can result in clinical disease.

Developmental Cycle

A unique biphasic developmental cycle in which chlamydiae alternate between the infectious elementary body (EB) and the vegetative reticulate body (RB) distinguishes chlamydiae from other intracellular bacteria. The EB is metabolically inert and resistant to the hostile extracellular environment, whereas the RB divides intracellularly and cannot survive outside the host cell.

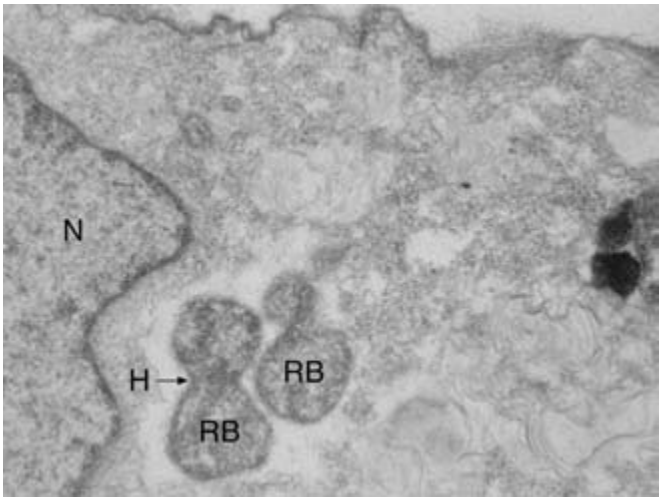


24.4. Buffalo green monkey (BGM) cell culture, 1 hour after inoculation with the *C. psittaci* Texas Turkey serovar D strain showing an elementary body (EB) attached to the side of a host cell microvillus.

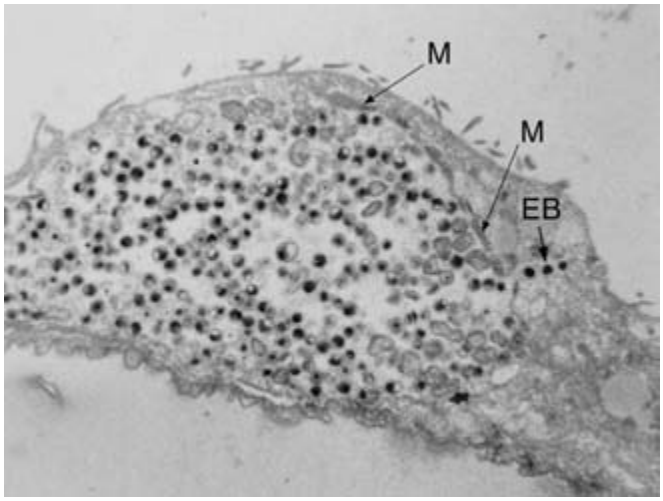


24.5. BGM cell culture, 1 hour after inoculation with the *C. psittaci* Texas Turkey serovar D strain showing an internalized elementary body (EB) within a membrane-lined vacuole. Notice the mitochondria (M) in the vicinity of the inclusion.

The initial event in the infectious process begins with attachment of *C. psittaci* EBs to microvilli at the apical surface of a susceptible columnar epithelial cell (51) (Fig. 24.4). The EB travels down the microvillus and locates in indentions of the eukaryotic plasma membrane, some of which resemble coated pits. The bases of micropilli represent areas of active transport of extracellular materials into the cells and, therefore, might assist rapid and efficient entry of EBs. After 1–3 hours, the EBs are internalized in invaginations of the plasma membrane (Fig. 24.5). Uptake of *C. psittaci* is an endocytic mechanism, involving microfilament-dependent and/or independent processes. The *C. psittaci*-containing endocytic vesicles or vacuoles escape interaction with

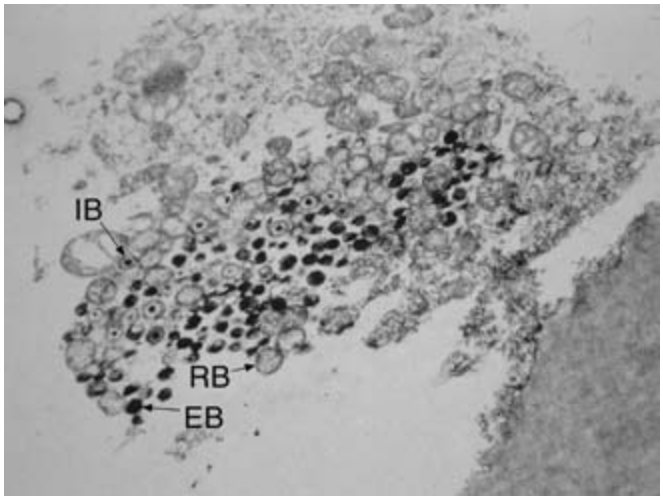


24.6. BGM cell culture, 18 hours after inoculation with the *C. psittaci* 89/1326 serovar B strain isolated from the spleen of a pigeon. Note the chlamydial vacuole near the nucleus (N) of the BGM cell with dividing reticulate bodies (RB) characterized by the "hour-glass" profiles (H).



24.7. BGM cell culture, 52 hours after inoculation with the *C. psittaci* 92/1293 serovar D strain, isolated from the lungs, the spleen, and the cloaca of turkeys. Note the large inclusion and the elementary bodies (EB) apparently "escaping" from the inclusion. Also notice the mitochondria (M) lining the inclusion.

lysosomes and proceed in about 8–12 hours to the cbn nuclear area, where EBs are converted to RBs. Conversion to RBs primarily involves reduction of disulfide bond cross-linking among the outer membrane proteins altering the EB cell wall. Synthesis of DNA, RNA, and proteins is initiated, permitting growth and binary fission of the RBs. Binary fission is characterized by the appearance of typical "hour-glass" profiles inside the vacuole (Fig. 24.6). The enlarging vacuole is also termed an "inclusion." *C. psittaci* microorganisms do not always remain within the inclusion throughout their intracellular development. In some cases and in apparent correlation with high pathogenicity of strains, the inclusion membrane seems to degrade during the active multiplication, liberating the bacteria into the cytoplasm of the host cell (97). About 30 hours after internalization of the EB, the first RBs are reorganized into newly formed EBs. At about 48–50 hours, the developing chlamydial inclusion may contain anywhere from 100–500 progeny, depending on the characteristics of the *C. psittaci* strain (Fig. 24.7). With most *C. psittaci* strains, the host cell has undergone severe degenerative changes, and microorganisms are released by lysis (Fig. 24.8). Exocytosis of the inclusion, followed by a "healing" or closing of the open-cavern structures where the inclusion had existed, has been reported (90). Persistent infections may occur with EBs remaining inside the host cell cytoplasm.



24.8 BGM cell culture, 50 hours after inoculation with the *C. psittaci* Texas turkey serovar D strain showing lysis of an infected BGM cell. Note the presence of reticulate bodies (RB), intermediate bodies (IB), and elementary bodies (EB).

Pathobiology and Epidemiology

Incidence and Distribution

Avian chlamydiosis occurs worldwide, with the incidence and distribution varying greatly with the species of bird and the serotype of the chlamydial organism. Psittacine birds harbor primarily one serotype of chlamydia that is endemic, and many psittacine birds are chronically infected. When under stress, chronically infected birds may become clinically ill or shed the organism. At these times, humans may become infected. The economic losses and the human infections usually follow a sporadic pattern; however, there are reports of outbreaks following the introduction of infected birds into pet stores or into homes. In recent years, antibiotics have been used extensively to control the spread of the disease in birds and to reduce the risk to humans. The pattern in pigeons appears to be similar, with at least two different serotypes being involved.

The disease pattern in turkeys appears to be changing. His-

torically, most outbreaks were explosive and occurred in free-ranging birds, but outbreaks now have been seen in confinement birds (104). Serotype D (virulent turkey) is usually found when death losses are high, whereas serotype B (pigeon) is less virulent (8). In these outbreaks chlamydia was usually thought to be introduced from the outside. However, recent studies on turkeys in Belgium and France showed that low virulent strains (serotypes A, B, E, and F) are widely distributed in commercial turkeys and may be endemic. Serology indicated that chlamydial activity is common, even though clinical signs are not seen. The chlamydial activity was usually seen prior to or during avian pneumovirus (APV) or ornithobacterium rhinotracheale (ORT) infections, indicating that chlamydia is part of the respiratory disease complex (92).

Information on the incidence and epidemiology of chlamydia in domestic ducks is limited. In the United States, it has not been a significant problem. In Europe, there have been a number of outbreaks, some of which have occurred in recent years (15, 57). Isolates from only a few European outbreaks have been serotyped, and these all have been of serotype C (103). This serotype also has been recovered from geese and swans.

Chlamydial strains from mammals are not a problem for poultry. Recent advances in serotyping using monoclonal antibodies and in strain identification using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) demonstrate that the strains occurring naturally in birds are distinct from those in mammals (7, 8). Attempts to infect birds with mammalian strains usually result in aborted or asymptomatic infections (53, 89). Avian strains will infect humans and may produce a severe pneumonia; however, secondary spread among humans rarely occurs (84).

Natural and Experimental Hosts

In addition to the naturally occurring chlamydial infections in domesticated birds, chlamydiae or chlamydial antibodies have been found in more than 400 wild avian species (1, 54). Common reservoirs of chlamydiae in the United States include wild and feral birds such as sea gulls, ducks, herons, egrets, pigeons, blackbirds, grackles, house sparrows, and killdeer, all of which freely intermingle with domestic birds. Highly virulent strains of *C. psittaci* can be carried by and excreted in large numbers by sea gulls and egrets without any apparent effect on these hosts.

Experimental hosts of avian chlamydiae can include virtually any species of bird, but it is thought that species vary in susceptibility. The length of time shedding and the numbers of chlamydiae shed may vary considerably depending upon the avian species. Antibody production as a consequence of a chlamydial infection also may vary.

Mammalian laboratory hosts used for avian chlamydiae are principally mice and occasionally guinea pigs. Both of these hosts can have naturally occurring chlamydial infections. Investigators using these animals should determine the chlamydial status of the breeding stock. Rabbits are refractory to clinical disease caused by avian chlamydiae, but they may be used to produce polyclonal antibodies (105).

Younger domestic birds generally are more susceptible than older birds to infection, clinical disease, and mortality. Infection in old turkeys, such as spent breeder hens, can go unnoticed unless birds are subjected to stressful conditions such as shipment to market on crowded trucks. Turkey toms may have a higher mortality rate than turkey hens.

Transmission, Carriers, and Vectors

The development of methods to readily serotype *C. psittaci* and to genetically characterize isolates is increasing the understanding of the epidemiology of the disease in birds. It is apparent that certain serovars of *C. psittaci* are usually associated with specific types of birds (e.g., serovar A with psittacine birds) and that some of these serovars have a parasite-like relationship with the host. That is, infection with *C. psittaci* will result in an inapparently infected carrier that under certain conditions such as stress will shed the organism. *C. psittaci* carrier status has been recognized in pigeons and psittacine birds and likely occurs with other birds. Shed chlamydiae recovered from psittacine birds is almost always serovar A and, from pigeons and doves, serovar B or E (8, 21, 103).

In turkeys, serovars associated with disease are not endemic but are introduced from feral birds. Isolates from large chlamydial outbreaks in turkeys are generally serovar B or serovar D (8, 103). Serovar B is common in pigeons and has been isolated from clinically normal pigeons and recovered from a number of turkey outbreaks with low mortality. Serovar D, usually found in high mortality outbreaks, has never been consistently associated with any avian species but turkeys; however, because of the methods used to raise turkeys and the time between outbreaks, it is doubtful that it is endemic in the turkey population. Most likely, another species of bird serves as the reservoir and transmission to turkeys occurs during commingling. The source or reservoir of serovar D is not known.

Chlamydial isolates of serovar C have been isolated from ducks and swans in Europe (100). Too few isolates have been serotyped to determine whether it is primarily a strain from ducks and other anseriformes. It is notable that this serovar has not been identified from other birds. In recent years, a few chlamydial isolates have been made from fatal cases in ratites. These isolates usually have been of serovar E, indicating that the ratites may be contracting it from pigeons or doves (4, 35).

Transmission of chlamydiae is probably by inhalation or ingestion of contaminated material. Large numbers of chlamydiae can be found in respiratory tract exudate and fecal material of infected birds. The importance of the respiratory exudate has become more apparent: In turkeys, the lateral nasal glands become infected early and remain infected for more than 60 days (89). Choanal/oropharyngeal swabs are more consistent for isolation of the agent than fecal swabs, especially during early stages of infection (6). Direct aerosol transmission through aerosolization of respiratory exudate must be considered as the primary method of transmission during outbreaks.

The role of arthropods in the transmission of chlamydiae is uncertain. Mites from turkey nests can contain chlamydiae (22), and during an epidemic in turkeys in South Carolina, simulid flies were suspected as a possible method of transfer (70).

Vertical transmission through the egg has been described for ducks, chickens, turkeys, and a number of wild birds (58, 100, 106). Its occurrence appears to be fairly low; however, it could serve as a method of introducing chlamydia into a flock.

Incubation Period, Clinical Signs, Morbidity and Mortality, Pathology and Pathogenesis

Turkeys

Page *et al.* (72) examined the pathogenesis of a *C. psittaci* infection in commercial turkeys after inoculation by aerosol or by the oral route with serovar D strain (8). Page determined the quantitative distribution of the organisms in tissues by isolation. Vanrompay *et al.* (98) studied the pathogenesis of *C. psittaci* serovars A, B, and D strains in specific-pathogen-free turkeys by immunodetection, which allowed the precise determination of the tissue and cell tropism. In this study, turkeys were aerosol infected as this most likely represents the natural route of infection as demonstrated by Page (72). From these studies, the following pathogenic sequence of events can be deduced for all three serovars investigated. In turkeys infected by aerosol, the primary site of replication is the upper respiratory tract, where epithelial cells become infected. Subsequently, epithelial cells of the lower respiratory tract and macrophages throughout the respiratory tract become infected. Then, intense replication occurs in the respiratory tract. At the same time, chlamydiae can be demonstrated in plasma and monocytes, indicating septicemia, and chlamydiae appear in epithelial cells and macrophages of various tissues throughout the body.

In turkeys, an experimental infection with a serovar B strain induced much milder clinical signs and lesions than infection with serovar A or D strains (101). Serovar B strain had a longer incubation period, took longer to reach maximum titers in the tissues, and had shorter periods during which the organism was found in the tissues.

The incubation period of chlamydiosis in naturally infected birds varies, depending upon the number of chlamydiae inhaled and the virulence or pathogenicity of the infecting strain for that host species. Experimentally, definitive disease signs in young turkeys receiving a virulent strain may be evident in 5–10 days. In birds naturally exposed to smaller doses or in older birds, the period may be longer. Strains of lower virulence, which cause less severe signs, may have longer incubation periods. Clinical signs may not be noticeable until 2–8 weeks after exposure.

Signs of chlamydiosis in turkeys infected with virulent strains are cachexia, anorexia, elevated body temperature, conjunctivitis, and respiratory distress. The birds excrete yellow-green, gelatinous droppings. Egg production of severely affected hens declines rapidly to 10–20% and may temporarily cease or remain at a very low rate until complete recovery. Disease signs in a flock infected with strains of low virulence are usually anorexia and loose, green droppings in some birds, with less effect on egg production.

At the peak of disease in a flock infected with a virulent strain, 50–80% of the birds will show clinical signs, and morbidity from less virulent strains is only 5–20%. Mortality caused by virulent chlamydia ranges from 10–30% and is only 1–4% with less virulent strains.

The less virulent strains cause gross lesions, which are similar to those caused by virulent strains, only less severe and extensive. In overwhelming infections with virulent strains, lungs show diffuse congestion, and the pleural cavity may contain fibrinous exudate. In fatal cases, a dark transudate may fill the thoracic cavity. The pericardial membrane is thickened, congested, and coated with fibrinous exudate. The heart may be enlarged, and its surface may be covered with thick fibrin plaques or encrusted with yellowish, flaky exudate. Severe damage to the lungs and heart undoubtedly is a major cause of death. The liver is enlarged and discolored and may be coated with thick fibrin. Air sacs are thickened and heavily coated with fibrinous exudate. The spleen is enlarged, dark, and soft and may be covered with gray-white spots representing areas of focal cellular proliferation. The peritoneal serosa and mesentery show vascular congestion and may be coated with foamy, white fibrinous exudate. All of these exudates contain large numbers of mononuclear cells in which numerous micro colonies of chlamydial RBs may be seen. Fibrinous exudates, found on all organs and tissues of the thoracic and peritoneal cavities, reflect vascular damage as well as increasing inflammatory response caused by the continued multiplication of the organisms.

In birds that survive infection with a strain of low virulence, the lungs may not be seriously affected. However, multiplication of organisms on the epicardium may result in the formation of one or more fibrin plaques.

Histopathologic changes in conventional turkeys of various ages injected intratracheally with the virulent Texas turkey (TT) strain of *C. psittaci* were first described by Beasley *et al.* (14). They observed both necrotizing and proliferative changes comparable to those caused by other chlamydial strains in other species (with the exception of focal necrosis of the liver, which is prominent in parrots and mice). Specific cellular changes and corresponding organ damage were decidedly more severe and extensive in young turkeys than in older ones. A majority of the birds examined had tracheitis characterized by extensive infiltration of mononuclear cells, lymphocytes, and heterophils in the lamina propria and submucosa. Cilia were absent in severely affected areas. This extensive tracheal damage is not necessarily characteristic of naturally infected birds and may be a result of intratracheal inoculation of large numbers of organisms. Epithelioid pneumonitis in varying degrees was found in 80–100% of 10-week-old birds but less often (10–20%) in mature birds. Lungs of severely affected birds were congested and had extensive infiltration of the tertiary bronchi and respiratory tubules with large mononuclear cells and fibrin. There was necrosis of individual cells and large areas of tissue; the parenchyma and stroma were equally affected. Fibrinous to fibrinopurulent inflammatory exudates were present on respiratory and peritoneal surfaces and on the epicardium in a majority of infected turkeys. The pericardium and epicardium were thickened by swelling of congested vessels and an inflammatory exudate containing fibrin, large mononuclear cells, and varying numbers of lymphocytes and heterophils. Myocarditis was observed in more than half the infected birds, but arteritis was present in only 8%. Hepatitis was present in more than 90% of the birds,

and in severely affected individuals, there was a diffuse dilation of sinusoids with infiltration of mononuclear cells, lymphocytes, and heterophils. Proliferated and swollen Kupffer cells were filled with debris, and a yellowish pigment thought to be hemosiderin. Necrotic hepatic cells were scattered throughout the organ with a few foci of necrosis. Acutely sick turkeys had a catarrhal enteritis. Spleens of a majority of birds were altered, with cellular proliferation and necrosis causing an enlarged and mottled appearance, which was more marked in younger than in older birds.

The organisms also caused orchitis and epididymitis, seeming to have an affinity for the active germinal epithelium (13). Fibrin and inflammatory cells appeared in association with the desquamated and necrotic epithelium, filling the seminiferous tubules with eosinophilic exudate. It was also observed that often the immediate cause of death in adult males was a rupture of testicular blood vessels followed by massive internal bleeding. The brains of 6 infected birds examined were without significant changes.

Vanrompay *et al.* (99) examined histopathologic changes in four groups of 20 specific-pathogen-free (SPF) turkeys, kept in isolation units and inoculated by aerosol. Turkeys were experimentally infected with strain 84/55 (*C. psittaci* serovar A), isolated from a parakeet, strain 92.1293 from a turkey (*C. psittaci* serovar D), the Texas Turkey strain (*C. psittaci* serovar D), or strain 89/1326 (*C. psittaci* serovar B) from a pigeon. All four strains of *C. psittaci* proved to be pathogenic for SPF turkeys. Turkeys showed conjunctivitis, sinusitis, rhinitis, keratitis, pneumonia, airsacculitis (Fig. 24.9A), pericarditis (Fig. 24.9B), hepatomegaly (Fig. 24.9C), splenomegaly, enteritis, congestion of the kidneys, and congestion of the ovaries or testes. Histopathologically, there were epithelial erosions and fibrin deposit in the conjunctivae (Fig. 24.10A), corneal ulceration, bronchopneumonia (Fig. 24.10B), fibrinous necrotizing airsacculitis (Fig. 24.10C), fibrinous pericarditis, interstitial nephritis, peritonitis, and catarrhal enteritis. The type and distribution of the lesions was similar for serovars A and D. The lesions produced after the serovar A infection, however, appeared more severe. For serovar B, in comparison with both other serovars, no lesions were observed in the small intestine, the pancreas, ovary, and testis.

Controlled dual infections in SPF turkeys demonstrated the pathogenic interplay between *C. psittaci*, APV, and *E. coli*. *E. coli* is a predisposing factor for the outcome of a *C. psittaci* infection. In its turn, *E. coli* can increase the severity of a *C. psittaci* infection and can reactivate a latent *C. psittaci* infection (91). An APV infection during the acute phase of a *C. psittaci* infection aggravates the severity of clinical signs, macroscopic lesions, pharyngeal APV excretion, and histological tracheal lesions. However, no clear interaction was established after APV infection in latently *C. psittaci*-infected turkeys (93).

Ducks and Geese

Chlamydiosis in domestic ducks is not an important disease in the United States, but it is important both economically and as a public health hazard in Europe. Chlamydiosis in ducks is usually a severe, debilitating, often fatal disease in which young ducks develop trembling, imbalanced gait, and cachexia. They become anorexic with green, watery intestinal contents. They develop a serous to purulent discharge from the eyes and nostrils causing the feathers on the head to become encrusted with exudate. As the disease progresses, the ducks become emaciated and die in convulsions. Morbidity ranges from 10–80%, and mortality varies from 0–30% depending on age and the presence of concurrent infection with salmonellae (87).

In recent years a number of outbreaks occurred in ducks in Europe and Australia in which the clinical signs in some outbreaks were minimal or absent (11, 15, 50, 57, 59, 66). Deaths and/or clinical signs were associated with stress of handling or with infection by other pathogens. Despite this change in pathogenicity, chlamydia in ducks has remained a public health problem.

Incidental to studies of chlamydiosis in ducks, several investigators have observed the disease in geese and have isolated *C. psittaci* from diseased tissues (87). Clinical disease and necropsy findings were similar to those in ducks.

Pigeons

The incubation period for chlamydiosis in pigeons is not known. Infection is endemic and is believed to be perpetuated primarily by a parent-to-nestling transmission cycle (20, 62).

Signs of uncomplicated chlamydiosis in pigeons are variable, but those that develop acute disease are anorexic, unthrifty, and diarrhetic. Some develop conjunctivitis, swollen eyelids, and rhinitis (Fig. 24.11). Respiratory difficulty is accompanied by rattling sounds. As disease progresses, birds become weak and emaciated. Recovered birds become carriers without signs of disease. Some birds progress through an infection showing no signs or, at the most, transient diarrhea before becoming carriers. Salmonellosis or trichomoniasis exacerbates the illness in chlamydia-infected carrier birds, inducing signs and lesions of acute disease. Serologic surveys indicate that infection rates in pigeons of 30–90%, and active infection rates, as measured by isolation, of 20% are common (61, 74, 82).

Gross lesions of uncomplicated chlamydiosis in pigeons are fibrinous exudates on thickened air sacs, the peritoneal serosa, and occasionally the epicardium. The liver is usually swollen, soft, and discolored. The spleen may be enlarged, soft, and dark. Greater than normal amounts of urates are seen in cloacal contents if catarrhal enteritis occurs. In less severe infections, only the liver or air sacs are involved. Some heavily infected shedders have no lesions (72, 74).

Chickens

Epidemiologic and laboratory evidence indicates that chickens are relatively resistant to disease caused by *C. psittaci*. Acute infection progresses to disease and mortality only in young birds, and the incidence of actual epidemics is very low. Experimentally, even young birds are resistant to many strains of *C. psittaci*. In acute cases, chickens have fibrinous pericarditis and hepatomegaly. Most natural infections in chickens are inapparent and transient; however, clinical cases with conjunctivitis, pericarditis, perihepatitis, and airsacculitis have been reported (10, 12).



24.11. Pigeons with no signs of chlamydial infection (top); moderate chlamydial conjunctivitis (middle); and severe chlamydial conjunctivitis (bottom).

Pheasants, Quail, and Partridges

Chlamydiosis has been reported in farm-raised pheasants, quail, and partridges. The clinical signs and lesions are similar to those seen in other birds. Morbidity and mortality can be very high, especially in young birds. Human infections have been reported (23, 40, 61, 87).

Immunity

Immunity to chlamydia is generally poor and short-lived. As birds become older, however, they become more resistant to clinical disease, even though infection may occur. Indeed, some birds, notably pigeons, are refractory to disease-producing infection even with highly virulent strains.

In turkeys, a moderate degree of resistance to organ damage is present by 15 weeks of age (13), and this resistence increases with further aging. The role active immunity has in preventing reinfection of naturally or experimentally infected turkeys has not been determined. The progress of infection initiated by an oral dose of chlamydiae and then spread by natural means through a group of 19 turkeys was followed by isolation attempts from blood and clinical observations (72). At varying times over a period of 47 days, each bird developed chlamydemia, hyperthermia, and mild anorexia. The chlamydemia lasted up to 10 days in each bird but was followed by clinical normalcy and apparent resistance to further bloodstream infection in spite of environmental contamination sufficient to infect all unexposed birds.

In ducks, resistance and immunity have not been sufficiently studied. Pigeons are apparently resistant to many avian strains of *C. psittaci*, even the highly toxigenic ones, but they are very susceptible to isolates from pigeons and sparrows (61, 71).

Despite extensive studies, the important host defense mechanisms against *Chlamydia* remain incompletely defined. An ongoing controversy is the relative contribution of humoral versus cell-mediated immunity in the host resistance against chlamydiae. What is known is primarily from studies in guinea pigs and mice.

In guinea pigs, both humoral immunity and cell-mediated immunity are essential for clearing the infection and for resistance against reinfection with the *C. psittaci* guinea pig inclusion conjunctivitis (GPIC) strain, currently classified as *Chlamydophila caviae* (76, 78, 79). However, in the murine system, T-cell-mediated immunity is evidently the critical immune mechanism for controlling *C. trachomatis* genital infection (52, 75, 77, 88).

Diagnosis

The methods used to diagnose *C. psittaci* infections are 1) direct visualization of the agent in clinical specimens by staining techniques, 2) isolation of the agent from clinical specimens followed by identification of the isolated agent, 3) detection of specific chlamydial antigens or genes in clinical specimens, and 4) serological tests in which antibodies are measured, preferably by demonstrating rising titers in paired acute and convalescent sera.

Specimen Collection and Direct Examination

Samples must be collected aseptically, especially for isolation, as contaminant bacteria can interfere with test results. At necropsy, the tissues of choice are air sacs, spleen, pericardium, heart, liver, and kidney. From live birds, choanal/oropharyngeal swabs and cloacal swabs are the first choices (6, 9).

If the samples are for isolation, proper handling is necessary to prevent loss of infectivity of chlamydiae during shipment and

handling. A transport medium consisting of sucrose-phosphate-glutamate (SPG) developed for *Rickettsia* is satisfactory for chlamydiae. The medium as recommended for chlamydiae consists of SPG buffer (sucrose 74.6 g/liter; KH_2PO_4, 0.512 g/liter; K_2HPO_4, 1.237 g/liter; and L-glutamic acid 0.721 g/liter) and can be autoclaved or filtered (86). To this is added 10% fetal calf serum and antibiotics. The transport media can be used as a diluent for freezing of chlamydia. The samples should not be frozen if they can be processed in 2–3 days.

Direct Visualization by Staining

Chlamydiae can be detected in smears and paraffin-embedded tissue sections by a number of techniques. The Gimenez, modified Gimenez (PVK stain), and the Giemsa staining techniques are commonly used (2, 3). The chlamydial EBs in this test will appear red against a green background.

Immunohistochemical staining is another method for detection of chlamydiae in cytological and histological preparations. This technique is more sensitive than histochemical staining, but interpretation of the results requires experience. The use of immunohistochemical staining of formalin-fixed sections is gaining popularity.

Isolation

Isolation attempts may be made by the inoculation of properly processed specimens onto cell culture monolayers, into the yolk sac of embryonated chicken eggs, or into mice.

Cell Cultures

Cell cultures are the most convenient method for isolating the avian strains of *C. psittaci*. McCoy, HeLa, Vero, L929, and BGM are the most commonly used cell lines, although a number of other cell cultures can be used. Standard tissue culture media and procedures, with a few modifications, are used. The laboratory equipment and supplies must be suitable to 1) stain and examine for chlamydial inclusions by direct immunofluorescence (IF) or other appropriate staining techniques, 2) permit centrifugation of the inoculum into the cell monolayer at 37°C or as near as possible, 3) permit staining and examination at two to three times during a passage, 4) permit blind passage at 3–4 days, and 5) provide protection of humans against possible infection. Small flat bottom vials (1 dram shell vials) or 24-well multi-well culture plates with 12 mm diameter glass cover slips will meet these requirements and are often used (2, 3, 43).

Suppression of cell division may be required to increase availability of nutrients for the growth of the chlamydiae and to permit longer observation of the infected cell cultures. The host cells can be suppressed by irradiation or by cytotoxic chemicals. Cytotoxic chemicals include 5-iodo-2-deoxygiodine, cytochalasin B, cycloheximide, and emetine hydrochloride (47). Cycloheximide is the most commonly used and is added to medium at a rate of 0.5–2.0 mg/ml at the time of inoculation of the monolayer. The effects of these drugs on replication of the chlamydiae can be variable; however, they appear to have no effect or a possible enhancing effect on the growth of the avian chlamydial strains.

Centrifugation of the inoculum onto the cell monolayer is often used to enhance the rate of infection. Centrifugation is routinely done at 500–1500 / g for 30–90 minutes. Temperatures near 37°C are preferred.

Tissue culture monolayers are fixed (with methyl alcohol, acetone, or an acetone-methyl alcohol mixture, depending on the staining technique and the cell culture vessel) and then stained and examined for inclusions on day 2 or 3 post-infection. The preferred method to demonstrate the chlamydial inclusion is the direct or indirect immunofluorescence staining using monoclonal antibodies against the genus-specific epitope on the chlamydial LPS or the MOMP (2, 3). The inclusion can also be demonstrated by cytological staining techniques, especially Gimenez, Stamp, Macchiavello's or Giemsa.

Chicken Embryo Inoculation

Fertile chicken eggs incubated 6 or 7 days at 39°C are inoculated with 0.2–0.5 ml/embryo via the yolk sac. Eggs for this use must be from chickens that are chlamydia free and that are not consuming chlamydiastatic antibiotics in their feed. Vascular congestion in the yolk sac is the predominant lesion seen in embryos dying from *C. psittaci* infection. The yolk sacs are harvested from embryos that die from 3–10 days after inoculation. If no embryos die, one or two blind passages should be performed. Yolk sac touch impressions are prepared for cytological staining or for direct or indirect immunofluorescence staining. The yolk sac harvest can also be used to inoculate onto tissue culture monolayers followed by identification of the chlamydial inclusions by the appropriate cytological or immunofluorescence stainings.

Detection of Specific Chlamydial Antigens

Tests that detect chlamydial antigen, as opposed to viable chlamydiae, have several advantages over isolation techniques. Antigen detection systems identify both viable and nonviable chlamydiae as well as soluble antigens in secretions or excretions. At present, direct immunofluorescence (IF), enzyme-linked immunosorbent assay (ELISA), and immunochromatography (IC) are available as rapid diagnostic tests for demonstrating chlamydial antigen. Most available tests use a monoclonal antibody (MAb) against the genus-specific epitope on the chlamydial LPS or against the species-specific epitope on the chlamydial MOMP and were originally developed for the detection of *C. trachomatis* or *C. pneumoniae* in human specimens. Some of these LPS-based tests have been evaluated for the detection of *C. psittaci* in avian specimens (16, 30, 55, 56, 102). The following conclusions can be drawn from these studies. The current rapid antigen detection methods are of relatively low cost and easy to perform, do not require rigorous maintenance of refrigeration for specimen shipment, and provide results much faster than culture. Of the antigen-detection tests evaluated in birds, at present the IMAGEN™ IF test seems to be the most specific and sensitive direct antigen detection test. However, IF is a diagnostic procedure with a sensitivity and specificity depending on the expertise of the observer. In general, the current generation of rapid diagnostic tests is not recommended for demonstrating *C. psittaci* in the individual bird because of shortcomings in sensitivity or specificity.

Detection of Specific Chlamydial Genes

C. psittaci-specific genes can be detected by both amplified and nonamplified nucleic acid-based detection methods. The polymerase chain reaction (PCR), as the most commonly used amplified nucleic acid-based detection method, focuses on the amplification of a portion of the *ompA* gene encoding the chlamydial MOMP or the 16S rRNA gene (26, 48, 49, 60).

Advantages of PCR for diagnosing chlamydiosis in birds are 1) easy, noninvasive sample collection methods, 2) simple transport and storage requirements, as viable chlamydiae are not needed, 3) avoidance of taking multiple samples as sometimes is recommended when using direct antigen detection tests, for instance ELISA, 4) rapid results, 5) high sensitivity, and 6) high specificity. In addition, production of targeted DNA fragments permits restriction endonuclease mapping of polymorphisms of the amplification product, thus facilitating species and strain differentiation (5, 24, 83, 96).

The main disadvantage of PCR is the risk of cross-contamination, necessitating strict operating procedures. False-negatives can occur through nucleic acid mutations in genes used as detection markers. Furthermore, positive PCR may sometimes reflect a carrier status. In those cases, quantitative PCR or serology may be helpful to confirm the clinical diagnosis of chlamydiosis.

Avian *C. psittaci* DNA amplification assays are performed only in specialized laboratories. Currently, PCR is used only in birds that are highly valued both monetarily and as companion animals. In the future, simplified cost-effective commercial PCR assays are likely to become available for routine chlamydial diagnosis in birds.

Serology

A diagnosis of chlamydiosis can be made by serology. This generally requires a four-fold increase in antibody titer on paired sera or a high number of positive titers in a flock. The complement fixation test is the standard and is used by most laboratories. Newer serological tests are available, some of which only detect IgM and are better indicators of current infection. Most of these tests require further evaluation by other laboratories before they can be recommended. For more information on the advantages of the various serological tests, the reader is referred to the following reviews and papers covering the tests (2, 3, 41).

Complement fixation (CF) is a widely used method for detecting anti-chlamydial antibodies. This is partly because the immunodominant carbohydrate-containing antigen of chlamydia readily induces complement-fixing antibodies. Such antibodies are no indication of immunity to reinfection with chlamydiae. They are useful, however, in detecting chlamydial infection, especially in a flock of birds. Generally, higher titers ($\geq$64 in poultry) are indicative of a current or recent infection. If low titers are obtained, it may be necessary to retest in about 10–14 days to detect any change in titer. A 4-fold or greater increase is considered to be diagnostic of a current chlamydial infection.

The direct and modified direct complement fixation procedures are the most commonly used to detect antibody in birds (43, 44, 45, 46). Both are relatively sensitive and can be performed using antigen prepared from cell culture-propagated chlamydiae. The modified direct procedure involves the addition of fresh chicken sera to the complement. This increases the sensitivity of the CF procedure so that it can be used to test sera from avian species whose antibodies do not normally fix guinea pig complement (42, 44).

The latex agglutination (LA) and elementary body agglutination (EBA) methods were developed for use with psittacine bird sera. However, the LA test is useful for screening turkey serum for antibodies and for testing pigeon and dove sera (39). Its usefulness for testing sera of ducks and geese is not known. The LA method predominantly detects IgG but will detect IgM (38). Its main disadvantage is that it is not as sensitive as direct CF, which detects only IgG activity in avian serum (38), nor as sensitive as the more recently described elementary body agglutination (EBA) test, which detects only IgM activity (36, 37). The EBA test has been evaluated and is currently being used for detecting antibody activity in various types of birds.

The indirect micro-immunofluorescence (IMIF) and indirect immunofluorescence (IFI) tests have been used extensively for serotyping of *C. trachomatis* in humans and more recently for identifying whether an immune response in humans is to *C. trachomatis*, *C. pneumonia*, or *C. psittaci*. The principles of the tests are similar in that both are an indirect IF test: the IFI test detecting reactions to inclusions in infected cell culture and the IMIF test detecting the EBs attached to a microscope slide. A recent report compared these tests to the CF and the ELISA for measuring antibody in pigeon sera and found the IFI and IMIF tests to be highly specific and efficient (82). The tests are used in serotyping avian isolates (8, 103) and have potential as a serological test. Older serological methods that are not widely used (e.g., rapid plate [or slide] and capillary tube agglutination, indirect CF inhibition, passive hemagglutination, immunodiffusion, and others) are described elsewhere (41). Newer methods such as indirect ELISA and indirect IF are being used, but they need to be thoroughly researched and evaluated to ascertain their usefulness.

Differential Diagnosis

Suspected chlamydiosis may have to be differentiated from pasteurellosis, particularly in turkeys, in which some signs and lesions may be similar. Pasteurellosis can be ruled out by appropriate culture procedures. Because of some similar signs and lesions, mycoplasmosis may need to be ruled out in turkeys suspected of having chlamydiosis. That can be accomplished by culturing and serologic testing for mycoplasmosis. Colibacillosis may mimic chlamydiosis to some extent; it can be excluded by the use of the appropriate coliform culturing procedures. Avian influenza may have to be ruled out in suspected chlamydiosis by virus isolation attempts and by serologic testing.

Intervention Strategies

Management Procedures

Ideally birds should be reared in confinement without any contact with potentially contaminated equipment or premises. Contact with potential reservoirs or vectors such as wild and feral

birds should also be prevented. General sanitation must be practiced diligently. Movement of people should be restricted so that visitors do not have free access to premises holding birds. This is easier to accomplish if birds are confined in houses.

Vaccination

Despite considerable efforts to develop an effective chlamydial vaccine, there have not been significant advances toward this goal. Thus, commercial chlamydial vaccines are not yet available.

Protective immunity to Chlamydiaceae is believed to be effected primarily through the action of CD4+ T helper type 1 (TH1) lymphocytes, CD8+ T lymphocytes, mononuclear phagocytes, and cytokines secreted by these cells. In addition, the role of local antibodies in mucosal secretions is not to be underestimated. The only protective chlamydial antigen that has been unambiguously identified is the major outer membrane protein (MOMP). However, attempts at chlamydial vaccine development based on a subunit design using MOMP have generally failed, probably because the immunogens did not induce the protective cellular and humoral immune responses elicited by native bacterial epitopes. In light of the current knowledge on protective chlamydial immunity, plasmid DNA expressing the MOMP of an avian *C. psittaci* serovar A strain has been tested for its ability to raise a protective immune response in SPF turkeys against challenge with the same serovar A strain. Good priming of T cell memory and protection against lung infection was observed (94, 95). Further studies are required to define the immune mechanism(s) for the protection observed.

Treatment

Treatments for poultry and pet birds have been reviewed (84, 100). Turkeys should be treated with chlortetracycline (CTC) at a concentration of 400 g/ton of pelleted feed. Care must be taken so that heat produced during the pelleting process does not destroy CTC and lower the concentration below an effective level. The CTC-medicated feed must be given for 2 weeks and then replaced by nonmedicated feed for 2 days prior to the birds being slaughtered for meat for human consumption. Calcium supplement should not be added to CTC-medicated pellets because calcium ions chelate CTC and diminish its effectiveness. It is recommended that all turkeys on an infected premise be treated and sent for slaughter: Reinfection can occur readily because resident wild birds may continue to harbor chlamydiae, or the treatment outlined may not rid all birds of chlamydia. In areas where chlamydiosis has been a problem, turkeys and ducks being marketed should be examined by a veterinarian and 1–2% should be tested serologically. It may be advisable also to attempt isolations on tissues from birds randomly selected from those tested serologically.

Essentially the same treatment methods are used to treat other fowl infected with *C. psittaci*. In other birds, salmonellosis may often be a complicating factor so it may be necessary to use a combination of antibiotics.

Pigeons should be treated with CTC-medicated feed, but the treatment may not be effective in eliminating the carrier state. Alternating periods of treatment with periods of no treatment may eventually clear the chronic infection (61).

State and Federal Regulations

State regulatory agencies may impose quarantine on intrastate movement of diseased flocks and may require antibiotic treatment of the flock prior to slaughter. Because regulations may vary from state to state, the appropriate public health and/or animal health agencies should be consulted as necessary.

According to USDA regulations, movement of poultry, carcasses, or offal from any premise is prohibited where the existence of chlamydiosis has been proved by isolation of a chlamydial agent. The Animal and Plant Health Inspection Service of the USDA and the U.S. Department of Health and Human Services forbid interstate movement of birds from infected flocks, but there is no restriction on movement of eggs from such flocks.

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III Fungal Diseases

25 Fungal Infections

Chapter 25

Fungal Infections

Bruce R. Charlton, R. P. Chin, and H. John Barnes

Introduction

Few fungal species are common pathogens in avian species, and these belong to the grouping of Eumycota (mycelial forms), as opposed to the Myxomycota (plasmodial forms) of the Kingdom Fungi. Fungi are unicellular or multicellular heterotrophic eukaryotes that derive nutrition by absorption; reproduce by asexual means, sexual means, or both; and possess cell walls. The Eumycota are composed of 5 phyla, or divisions. Four divisions are differentiated by the morphology of sexual structures; the fifth, Fungi Imperfecti (Deuteromycota), is distinguished by the lack of evidence of a sexual phase (1).

Classification of fungi historically has been based on morphologic characteristics of the sexual form (teleomorph) and asexual form (anamorph), with the teleomorph state assuming precedence in nomenclature, under the umbrella of Kingdom Plantae. The result is at times confusing, or even crosswise of common sense, as for instance, the nomenclature applied to aspergilli. The genus *Aspergillus* belongs to the division Deuteromycota, which is composed entirely of anamorphs; however, some aspergilli also have teleomorphs, and so by definition, they belong to a separate division and are assigned to a different genus as well. For example, *Aspergillus fisherianus* the anamorph becomes *Neosartorya fischerianus* the teleomorph by meitotic reproduction of ascospores (division Ascomycota). In addition, reproductive patterns aside, synonyms have been applied lavishly to certain fungal species and diseases over time. An attempt is made in this chapter to present taxonomy that conforms to the International Code of Botanical Nomenclature (Tokyo Code).

Mycoses are relatively uncommon diseases but are often devastating to the host supporting the fungal infection. The ability of fungi to infect animals is incidental to their maintenance. Propagation and dissemination occur as a result of their saprobic lifestyle; infection is a dead end, with the exception of dermatophytoses, because mycoses are not contagious.

The chapter is organized like the previous edition. The first focus is aspergillosis, followed by other fungal infections representing the less common mycoses. Aspergillosis is by far the most commonly encountered mycosis of birds and is an economically important respiratory disease of poultry. Candidiasis is the principal fungal infection of the digestive tract of poultry. Dermatophytosis affects the integument and is the only contagious and zoonotic mycosis. Some rare fungal infections of poultry are briefly discussed; histoplasmosis and cryptococcosis are notable as public health hazards.

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Aspergillosis

Introduction

Definition and Synonyms

Aspergillosis is defined as a disease caused by infection with the genus *Aspergillus*, which is composed of approximately 600 species (103). Manifestations of aspergillosis depend upon which organs or systems are involved and whether infection is localized or disseminated. Aspergillosis in birds is usually confined to the lower pulmonary system with florid lesions in air sacs and lungs. In young poultry, the disease is referred to as brooder pneumonia. Other synonyms for avian aspergillosis include fungal or mycotic pneumonia, pneumonomycosis, bronchomycosis, and colloquialisms such as “asper” and “air sac.” Less common manifestations relate to infections of the eye, brain, skin, joints, and viscera.

Economic Significance

The financial cost of aspergillosis is most readily apparent in turkey production where the disease primarily affects grow-out flocks. In Iowa, between 1985 and 1994, aspergillosis was reported in a yearly average of 8.3% of flocks ranging in age from 13–18 weeks (76). Mortality attributed to aspergillosis averaged 4.5%, placing it as the second most costly disease with an esti-

This chapter is a revision of previous editions authored by R. A. Kunkle, H. L. Chute and J. L. Richard, respectively. The bulk of information presented herein is a result of their exemplary research, literature sleuthing, and expert authorship.

mated yearly loss of $5,260 per affected flock in Iowa—$338,000 annually statewide. In 2000, Iowa ranked ninth in turkey production in the United States with roughly a 3% share of national production (10). Assuming an even distribution of aspergillosis across the country, mortality losses from the disease results in financial losses of more than $11 million yearly nationwide. However, losses from carcass condemnations typically far outstrip mortality losses.

Public Health Significance

Aspergillosis is not a zoonotic or contagious disease. Allergic pneumonitis can develop in those with frequent exposure to moldy hay, compost, or other forms of decaying organic matter in which *Aspergillus* and other allergenic organisms thrive. Severely immunocompromised individuals are at risk for developing opportunistic infections, including aspergillosis.

History

Molds, likely belonging to the genus *Aspergillus,* were described in wild birds in the early 1800s occurring in such species as the Scaup duck, jay, and swans (6, 92). The first time that an *Aspergillus* was described in a lesion, however, was in 1842 when Rayer and Montagne (86) identified *A. candidus* from the air sac of a bullfinch. *Aspergillus fumigatus,* the most frequently observed agent of avian aspergillosis, was first found in the lungs of a bustard (*Otis tardaga*) in 1863, and the species name was attributed to Fresenius (18). He also applied the term *aspergillosis* to this respiratory disease. Interestingly, early investigators believed that fungi found in lesions of avian species were growing saprophytically on "morbid products" in the body (6). Aspergillosis is common in turkey poults, having been described by Lignieres and Petit (61). Hinshaw (43) described the disease in adult turkeys.

Etiology

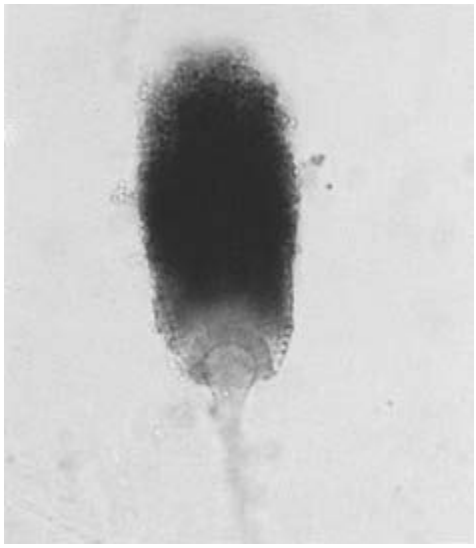
Classification

The principal agent causing aspergillosis in poultry is *Aspergillus fumigatus*. Isolation of *A. flavus* is less common. Other species rarely isolated include *A. terreus, A. glaucus, A. nidulans, A. niger, A. amstelodami,* and *A. nigrescens*. Both *A. fumigatus* and *A. flavus* lack a sexual stage and require only one classification scheme: division Deuteromycota, class Deuteromycetes, order Moniliales, family Moniliaceae, genus *Aspergillus*. Alternate classification schemes for the genus appear to be equally valid (85).

A. fumigatus *Fresenius 1850*

Colony Morphology

The organism grows rapidly on Sabouraud dextrose, Czapek's solution, or potato dextrose agar (25–37°C) with colonies having a diameter of approximately 3–4 cm in 7 days. The flat colonies are white at first and then bluish green as conidia begin to mature, especially near the center of the colony. As the colony matures, the conidial masses become gray-green, and the colony edge remains white. The colony surface varies slightly among isolates being either smooth and velvety to slightly floccose or folded. The colony reverse is usually colorless. The description presented here includes the most typical characteristics of clinical isolates, but variations occur in colony color, morphology, and growth rate (60).



25.1. Conidiophore with flask-shaped vesicle, phialides, and columnar mass of chains of conidia of *Aspergillus fumigatus.* ×250.

Microscopic Morphology and Staining

Hyphae measure 3–7 μm in diameter, have parallel sides, are septate, and branch dichotomously. The conidiophores, spore-bearing branches of *A. fumigatus,* are smooth, colorless to light green near the vesicle, up to 300 μm in length, and 5–8 μm in diameter (Fig. 25.1). The conidiophore gradually enlarges distally to form a flask-shaped vesicle. The vesicle is 20–30 μm in diameter with a single series of phialides (conidiogenous cells) over the distal half. The phialides (6–8 μm in length) are arranged upward paralleling the axis of the conidiophore. A distinctive feature of *A. fumigatus* is the development of columnar masses of chains of conidia arising from the vesicle. The conidial chains may attain a length of up to 400 μm. The conidia, green in mass, are echinulate, globose to subglobose, with a diameter of 2–3 μm.

Visualization of wet-mount specimens is aided with methylene blue or lactophenol cotton blue stains. Localization of fungal elements in embedded tissue sections is facilitated with periodic acid-Schiff or Gomori's methenamine silver stains.

A. flavus *Link 1809*

Colony Morphology

The organism grows very rapidly, obtaining a colony diameter of 6–7 cm in 10 days at 25°C on Sabouraud dextrose, Czapek's solution, or potato dextrose agar. Some isolates may be slower growing. The colonies begin as white, close-textured mycelium, turning yellowish to yellow-green with a white colony edge as



25.2. Conidophore with globose vesicle. Phialides and radiate chains of conidia of *Aspergillus flavus*. ×250.

conidia develop. Mature colonies may become somewhat olive-green. The colony may be furrowed radially or flat. Brownish to black-brown sclerotia (densely tangled mats of mycelia), which begin as white tufts of mycelium, may be more evident than conidial development in some isolates. Isolates vary considerably in color and number of sclerotia, if any are present. The colony reverse varies from colorless to pinkish drab to brownish in sclerotial strains. The conidial heads of *A. flavus* are radiate with the chains of conidia splitting to form loose columns.

Microscopic Morphology

The conidiophores (up to 100 μm long and 10–65 μm in diameter) of *A. flavus* are thick-walled, rough, and colorless. The vesicles, although more elongated when young, are globose to subglobose (10–65 μm in diameter) with phialides usually in 2 series (biseriate or 2-layered) on the entire surface of the vesicle (Fig. 25.2). Phialides may be one series (uniseriate), or, more rarely, each condition may be present in a single head. The conidia are globose to subglobose, echinulate, and between 3 and 6 μm in diameter (usually 3.5–4.5 μm).

Growth Requirements

The causative agents of aspergillosis are ubiquitous, due in part to their indifferent nutrient requirements. They commonly occur in soil, grains, and decaying plant matter. Growth is supported in virtually all laboratory media but is inhibited by cycloheximide. *Aspergillus fumigatus* grows well with chicken feather keratin as its sole carbon and nitrogen source (104) and can colonize fiberglass by using binding resins for nutrition (31). The optimum temperature for rapid culture of *A. fumigatus* is 40°C; however, few environments impede growth. The organism is thermotolerant, with growth occuring at temperatures as high as 55°C and survival maintained at temperatures up to 70°C (59). Growth also occurs at air temperatures as low as 9°C and at relative humidity levels ranging from 11–96% (77). An oxygen tension as low as 0.5% will support growth and conidiation (40).

Biochemical Properties

Identification of fungal agents, including the aspergilli, are based primarily upon colony and microscopic morphology and growth characteristics; biochemical criteria are used infrequently as adjuncts to species identification. Analysis of isoenzyme patterns has been examined as a taxonomic tool. The only enzyme pattern common to all strains of *A. fumigatus* is that of glutamate dehydrogenase (59).

Susceptibility to Chemical and Physical Agents

Some species of the genus *Aspergillus* appear to be quite resistant to chemical agents and have been known to occur in sanitizing fluids, sulfuric acid, copper sulfate plating baths, and formalinized tissues for museum specimens (103). Phenolic disinfectants are commonly used fungicidal agents. Commercial preparations of enilconazole have been used to control aspergilli in the poultry house environment (88). Certain oils derived from spices, such as cinnamaldehyde, are inhibitory to *Aspergillus* growth (64).

Antigenic Structure and Toxins

Antigens

The aspergilli produce a range of antigenic molecules. The antigenic and allergenic properties of *A. fumigatus* extracts have long been recognized in the field of mammalian aspergillosis, but few reports have been made of the serologic responses of birds infected with *Aspergillus*. The qualitative and quantitative variability of the composition of the antigenic extracts, even between batches prepared in the same laboratory, and the multitude of antigens produced increase the difficulty in preparing standardized reagents (42, 59).

Antigens prepared from *A. fumigatus* and *A. flavus* have been used in the detection of antibodies in experimentally exposed turkey poults (78, 98) and chicks (115). The preparations used in the poult studies were culture filtrate antigens produced on either a neopeptone dialysate medium or on Dorsett's medium (117).

Allergic skin reactions in avian species against an alcoholic precipitate from a mycelial extract of *A. fumigatus* have been studied (5). Penguins showed severe and prolonged skin sensitivity, whereas pigeons and ducks were quite resistant. Zoo penguins frequently die from *A. fumigatus* infections.

Detection of the immunodominant galactofuranosyl residues of galactomannan (12), a major cell wall component of *A. fumigatus* (91), in sera or urine with immobilized monoclonal antibody (113) has proved useful in aiding early diagnosis of invasive pulmonary aspergillosis in humans. However, the antigen is not specific for *Aspergillus*, and the occurence of galactomannan in foods and antibiotics may lead to false-positive results (2). The applicability of using galactomannan antigenemia to diagnose aspergillosis in poultry is untested.

Monoclonal antibodies to a 106-kD cell wall antigen of *Asper-*

gillus species were developed for immunohistochemical examinations of archived paraffin-embedded tissues (48). The commercially available MAb-WF-AF-1 works well to identify *A. fumigatus* conidia and hyphal elements in experimentally infected turkey tissues.

Toxins

Aspergillus spp. are among the 3 most common mycotoxigenic genera. Aflatoxins, along with other mycotoxins, are discussed in detail in Chapter 33.

Numerous experimental studies have demonstrated that toxins consumed by poultry can interfere with resistance to various infections. A concept that is not well-recognized, however, is that the pathogenic species of *Aspergillus*, particularly *A. flavus* and *A. fumigatus,* produce toxins that could be involved in the pathogenesis of aspergillosis in poultry. Richard *et al*. (98) found no enhanced pathogenicity of aflatoxigenic strains of *A. flavus* in turkey poults. *A. fumigatus* conidia, however, caused approximately 50% mortality and induced antibodies in poults exposed by aerosol; whereas *A. flavus* conidia caused neither mortality nor antibody production. Some authors had considered previously that a toxin was involved in avian aspergillosis caused by *A. fumigatus* (110). Extensive necrotic lesions (98) and torticollis observed in turkey poults in the absence of brain lesions (99) were reasons for consideration of toxin involvement in aspergillosis caused by *A. fumigatus* (102).

Gliotoxin is one of several toxins produced by various isolates of *A. fumigatus*; it was found to be produced by most of the isolates obtained in an outbreak of aspergillosis of turkeys (93). Turkeys are quite sensitive to oral doses of the toxin. Gliotoxin also is immunosuppressive; it is cytotoxic to, and inhibits blastogenesis of, turkey peripheral blood lymphocytes (101). Richard and DeBey (95) reported the presence of gliotoxin in excess of 6 ppm in tissues of experimentally infected turkey poults, and higher concentrations of gliotoxin have since been found in turkey lung tissue of naturally occurring cases of aspergillosis (96). In a murine model of disseminated aspergillosis, animals infected with a nongliotoxin producing strain survived significantly longer than those infected with a gliotoxin producing strain of *A. fumigatus* (114). It is concluded that gliotoxin production contributes to the pathologic changes observed with aspergillosis.

Virulence Factors

Metabolites, which are necessary for the pathogenicity of *A. fumigatus*, have not been identified. A discussion of the organism's virulence factors requires consideration of its success as a saprobe, in addition to its role as an accidental, noncontagious pathogen. The ability to use a variety of substrates, the copious production of spores that are easily airborne, and the hydrophobicity of the conidia all contribute to its achievement as an ubiquitous saprobe. The widespread distribution of the organism ensures inhalation of conidia by susceptible hosts. The conidia are small enough, 2–3 μm in diameter, to bypass the physical barriers of the upper respiratory tract and are deposited deep in the pulmonary system. The accelerated growth rate of the fungus at temperatures found within piles of rotting vegetation coincides with the body temperature of homeotherms. *In vitro* growth of *A. fumigatus* is enhanced by the presence of hydrocortisone, the pharmaceutical equivalent of the endogenous stress-induced hormone cortisol (71). The occurrence of aspergillosis is a function of the inhaled dose of conidia and the susceptibility of the host.

A. fumigatus produces a number of proteolytic enzymes that can degrade host tissues, especially components of the extracellular matrix. The elastinolytic and collagenolytic enzymes are of particular interest; however, studies using knock-out mutants have not yet identified proteases, which play an essential role in the pathogenesis of pulmonary aspergillosis (59).

Pathogenesis and Epidemiology

Incidence and Distribution

Primarily two forms of aspergillosis occur in poultry. Acute aspergillosis usually is characterized by severe outbreaks in young birds and high morbidity and high mortality. Chronic aspergillosis occurs in adult breeder birds (particularly turkeys) or occasionally in birds in an adult flock or aviary. The incidence of chronic disease is not as great, but in commercial poultry flocks, there are significant economic losses when adult birds succumb to this disease. Aspergillosis appears to be more significant in confinement situations where stress factors may be involved or where moldy litter or grain is present.

Contaminated poultry litter is often the source of *Aspergillus* conidia (29). Pinello *et al*. (82) isolated 73 species of fungi from air, litter, and tissues in a confinement turkey house, and *Aspergillus* was among the 4 major genera found. *Aspergillus* spp. were among the most common fungi found in other studies of air or litter flora of poultry houses (53, 63, 106, 116). Air flora density of the 4 major genera within the poultry house decreased when the windows were opened during the spring (100). Reducing the dust in poultry houses and improving ventilation resulted in a 75% decrease in the incidence of fungal disease (89). Elimination of moldy feed from the diet and environment, along with the proper management of the sawdust litter, prevented reoccurrences of fungal ophthalmitis in poultry houses where there was a history of such problems (11).

Outbreaks occur when the organism is present in sufficient quantities to establish disease or when the bird's resistance is impaired by factors such as environmental stresses, immunosuppressive compounds, inadequate nutrition, or other infectious diseases.

Chute *et al*. (22) observed that *A. fumigatus* is found frequently and is not always pathogenic in young broiler chicks. Sixteen isolates of *A. fumigatus* were compared for pathogenicity by air sac inoculation of turkey poults. Mortalities were not influenced by the number of conidia given or the source of the isolates, although a single environmental isolate produced no mortality (78). Chute and coworkers (22) found the following genera in lungs and air sacs: *Aspergillus, Penicillium, Paecilomyces, Cephalosporium, Trichoderma, Scopulariopsis,* and *Mucor.*

Aspergillosis can be one of the most frequently reported diseases and a source of considerable monetary loss in turkeys (75, 76).

Natural and Experimental Hosts

Aspergillosis occurs in most domesticated and several wild animal species. Mycotic abortion caused by *A. fumigatus* is an economically important disease of dairy and beef cattle worldwide. Infection in horses typically is manifested as guttural pouch mycosis (81). Poultry, exotic, and wild avian species appear to be particularly susceptible to pulmonary aspergillosis. Aspergillosis in humans has increased dramatically in incidence over recent years as a complication of therapeutic immunosuppression (8). Immunocompetent mammals, unlike birds, are naturally resistant to pulmonary aspergillosis unless exposed to an overwhelming dose of conidia. Rodents used as models for the study of invasive pulmonary aspergillosis must be pretreated with cortisone or another immunosuppressive agent for the disease to develop (70). In contrast, experimental aspergillosis in turkeys requires no pretreatment to confer susceptibility (54).

Age of Host Commonly Affected

Pulmonary aspergillosis is the most common form of the disease in avian species and is seen most frequently in young broiler chickens, turkey poults, young and mature breeder turkeys, captive raptors, and penguins (81). The disease is known to occur in a wide variety of avian species, and perhaps all birds, captive and free, domesticated and wild, should be considered as potential hosts susceptible to *Aspergillus* infection.

Pulmonary Aspergillosis

Experimental pulmonary aspergillosis was produced readily by intrathoracic injection of fungal conidia as early as 1884 (119). In 1935, Durant and Tucker (28) produced the disease in a poult by feeding mash contaminated with *A. fumigatus.* Ghori and Edgar (36) found differences in susceptibility to *A. fumigatus* among Japanese quail, turkeys, and chickens, and also strain differences among three strains of chickens (37). Inbred strains were more susceptible than crossbred or outbred strains during an outbreak of aspergillosis in hatchery chicks (14).

Penguins appear to be extremely susceptible to aspergillosis (3), but the disease is significant primarily in captive penguins (52, 72).

Three-to-eight-week-old ostriches in Israel were found to have pulmonary aspergillosis as a dual infection with *A. flavus* and *A. niger* (79). These 2 organisms were isolated from all samples of affected lungs.

Systemic Aspergillosis

Systemic aspergillosis in poults was reported by Witter and Chute (123). Chute *et al*. (21) also reported systemic aspergillosis infection in caponized 5-week-old cockerels. The authors concluded that this resulted from a caponizing infection. An outbreak of *A. flavus*-induced systemic aspergillosis in turkey poults with sternal bone involvement was described by Ghazikhanian (35). Intravenous inoculation of *A. fumigatus* conidia produces acute miliary hepatitis (57).

Dermatitis

Cutaneous lesions as a manifestation of aspergillosis are rare in avian species. Necrotic granulomatous dermatitis was described in chickens, and *A. fumigatus* was isolated from infected tissue (126). Lahaye (58) discussed cutaneous aspergillosis of pigeons.

Omphalitis

Simultaneous *A. fumigatus* infection of the yolk sac and lungs of young turkey poults has been reported by Cortes *et al*. (24). Increased morbidity and mortality were observed in birds between 3 to 9 days of age. Culture of the organism and demonstration of mycelial invasion of the tissue by histopathology are required to establish a diagnosis.

Osteomycosis

A. fumigatus infection in bone caused deformed vertebrae resulting in partial paralysis of young chickens (13). Presumably, the infections were sequelae to lung disease with hematogenous dissemination of the organism. Note that the outbreak of systemic aspergillosis described previously (35) included involvement of the sternal bone and was caused by *A. flavus.*

Ophthalmitis

Ophthalmic lesions in birds due to *Aspergillus* (Fig. 25.3A) have been reported since 1940 when Reis (90) recognized aspergillosis in the chicken eye. Similar lesions were described by Hudson (44) in young chicks and by Moore (69) in turkey poults. Although these cases of ophthalmitis in avian species were similar in that the infection was unilateral, an important difference occurred in these early reported cases. The first 2 cases had involvement primarily of the conjunctiva and external surfaces of the eye with the development of a cheesy exudate or plaque forming beneath the nictitating membrane. The fungus could be isolated readily from cultured plaque material. The eye infection described by Moore (69) involved turkey poults that had respiratory aspergillosis, and the cornea of the eye was not involved. Most of the pathologic changes occurred in the posterior eye involving the vitreous humor and extending into adjacent tissue. Thus, the pathogenesis of the 2 conditions was apparently quite different; the keratitis and superficial infection probably resulted from exposure of conjunctival surfaces to viable fungal elements from environmental sources. The fungal ophthalmitis involving the posterior eye, however, may have resulted from a hematogenous or lymph dissemination of the organism from a primary respiratory infection. Although not a frequent occurrence, the latter type of eye infection usually is apparent in birds with respiratory involvement. Reis (90) was able to reproduce a superficial eye infection in chickens by introducing conidia of *A. fumigatus* into the eye. The yellow caseous plaque can become adherent to the cornea in the superficial type of infection (47). Because of swelling, this infection may resemble coryza or vitamin A deficiency in chicks (112). After Chute and O'Meara (20) injected conidia of *A. fumigatus* into the abdominal air sacs of chickens, one bird developed a plaque on the surface of one eye. They did not speculate concerning the route of infection.

Recently, Beckman and coworkers (11) described a somewhat different fungal ophthalmitis in a group of chicks from a breeder farm that had recurring problems with similar eye infections. The causative agent was *A. fumigatus*, but in addition to exudate

within the conjunctival sac, there was histopathologic evidence of fungal invasion of the anterior chamber and cornea similar to that described by Moore (69). Although the chicks had respiratory lesions, the authors did not consider the route of infection to be hematogenous from the respiratory lesions, because there was no involvement of intraocular structures.

Occasionally, turkeys experimentally exposed to aerosols of conidia developed a cloudy eye with retinitis, iridocyclitis, and secondary involvement of the remainder of the eye (100). There was a cellular infiltration of heterophils and macrophages, and cellular debris and fungal elements were present in the chambers and retina. The pecten was severely involved with edema, heterophils, mononuclear cells, and fungal elements present. In some turkeys, the pecten contained granulomas.

Following oculonasal vaccination against Newcastle disease, mortalities increased rapidly in chickens that had contracted superficial ocular aspergillosis in the hatchery (66). This type of ocular involvement was described by Moore (69) and occurred in 5 widely separated flocks of young poults and in 3 breeding flocks.

Encephalitis

Numerous reports have described encephalitic or meningoencephalitic aspergillosis in a variety of avian species. In turkeys, necrotic foci in the cerebrum or cerebellum were found in naturally occurring aspergillosis (84). Richard *et al*. (99) found such foci in turkey poults experimentally exposed to aerosols of *A. fumigatus* conidia. Outbreaks of meningoencephalitis have occurred in turkey poults, eider ducklings, and chickens (127). Others have described encephalitic aspergillosis in turkey poults and chickens occurring as caseous necrotic lesions of the cerebrum and cerebellum or as granulomatous encephalitis (1, 38).

Jungherr and Gifford (51) found fungal hyphae in the cerebellum of a poult that had exhibited nervous symptoms. In another outbreak in poults with pneumonomycosis and nervous manifestations, they recovered *A. fumigatus, A. niger,* and *Penicillium varioti* from internal organs. Bullis (15) recovered *A. fumigatus,* and later *Diplococcium* spp., from the cerebra of poults that showed incoordination. In most cases, concurrent infection of the lungs and air sacs occurred, and often the kidney and liver were involved.

Transmission, Carriers, and Vectors

Aspergillosis is not a transmissible disease. Infections are acquired from environmental exposure. Disturbances of soil or movement of hay, compost, or litter can produce aerosols that furnish occasion for respiratory exposure to conidia. Fresh litter contaminated with *A. fumigatus* can precipitate outbreaks of aspergillosis (29).

Aspergillosis can also be acquired *in ovo*. A case of egg-borne aspergillosis was reported by Eggert and Barnhart (30). They suggested that the fungus had penetrated through the eggshell during incubation, and recently hatched chicks were infected. Clark *et al*. (23) reported on other cases of aspergillosis that originated in hatcheries. From 21 ranches where 210,000 chicks were involved, there was mortality of 1–10%. Infection could not be traced to hatching eggs but was readily found in incubators, hatchers, incubator rooms, and intake ducts. Signs and lesions were noted in some day-old chicks, but generally, classic lesions were observed in chicks 5 days of age.

O'Meara and Chute (73) found that hatching chicks and up to 2-day-old chicks were infected easily with *A. fumigatus* spores by contaminating the forced-draft incubator with wheat seeded with *A. fumigatus.* Chicks older than 3 days were resistant to infection.

Egg embryos are quite susceptible to infection by *A. fumigatus* during incubation. Embryo contamination occurred when a petroleum jelly suspension of *A. fumigatus* conidia was applied to the surface of incubating eggs (124), and infections increased when the incubating eggs were dusted with *A. fumigatus* conidia (125). Within 8 days after the dusting application, the organism had penetrated the eggshell.

Incubation Period

In an outbreak in captive wild poults, mortality totaling 75% began at 5 days, reached a peak at 15 days, and subsided at 3 weeks of age (28). Some affected poults died in convulsions within 24 hours. Onset of signs did not occur before 48 hours in turkey poults experimentally infected with high doses of *A. fumigatus* or *A. flavus* (100). Airsacculitis can be evident within 24 hours of intra-air sac inoculation of *A. fumigatus* (54).

Signs

Signs can be subtle, even in cases in which post-mortem examination reveals severe airsacculitis. Dyspnea, gasping, and accelerated breathing may be present. When these signs are associated with other respiratory diseases, such as infectious bronchitis and infectious laryngotracheitis, they often are accompanied by gurgling and rattling noises, whereas in aspergillosis there usually is no sound. Guberlet (39) ascribed somnolence, inappetence, emaciation, increased thirst, and pyrexia to aspergillosis. Cases under his observation emaciated rapidly and showed diarrhea in the later stages. Dysphagia was noted in cases in which esophageal mucosa was involved. Mortality was as high as 50% in confined birds on some farms, whereas birds running outdoors were more resistant or escaped infection entirely. According to Van Heelsbergen (119), some workers reported serous excretions from nasal and ocular mucosa. Extreme dyspnea was recorded in canaries by De Jong (26). Gauger (34) reported an outbreak of aspergillosis in adult chickens in which about 10% of the flock had signs resembling laryngotracheitis, but there was no increased mortality, although egg production was temporarily lowered.

Because torticollis and/or a lack of equilibrium occurs in both experimental (99) and in naturally occurring infections by *Aspergillus* spp. (84, 120), this should be considered as a sign of avian aspergillosis. However, other infectious agents, including other genera of fungi, can cause similar signs.

Morbidity and Mortality

Aspergillosis is characterized by high morbidity and mortality in chicks and poults and by low morbidity and mortality in mature poultry. Morbidity can be underestimated in finishing flocks until slaughter inspection reveals pulmonary lesions. Condem-

nation of mature young turkey carcasses due to airsacculitis is the second leading cause of post-mortem condemnations in the United States (83).

An outbreak of aspergillosis associated with contaminated litter resulted in the loss of approximately one-third of a flock of turkey poults (29). The litter may have supported fungal growth during the outbreak. Mortality of 96% of a poult flock was recorded in another outbreak; the possible connection of the addition of glucose to drinking water, and the source of infection was not addressed (74). A change of feed and litter may have helped quell an outbreak in chickens which resulted in 26% mortality (67).

Approximately 50% of turkey poults died following a 10-minute aerosol exposure to conidia of *A. fumigatus*, resulting in 5×10^5 colony-forming units/g of lung tissue (98). No deaths occurred in turkey poults similarly exposed to *A. flavus,* perhaps because the size of *A. flavus* conidia (3–6 μm) is considerably greater than that of *A. fumigatus* (2–3 μm) and, thus, they would not reach as deeply into the respiratory tract. Walker (121) reported that 5–7-day-old ostriches succumbed in 2–8 days from pulmonary aspergillosis after conidia were aerosolized into the trachea. Turkey poults aerosol-exposed to 2.2×10^6 viable units of *A. fumigatus*/g of lung tissue all died by day 5; lower doses (5.2×10^5 viable units) delayed and reduced mortality. Deaths began by 3–4 days postexposure.

Gross Lesions

The lesions of uncomplicated pulmonary aspergillosis evolve over several days and diminish in a few weeks time. The acute lesions of experimental aspergillosis in turkeys rapidly progressed in severity. At 24 hours, white miliary foci were present on air sac membranes, and lung lesions consisted of straw-colored gelatinous subpleural edema. Air sacs became progressively thicker and opaque and supported granulomata that increased in size and changed shape from raised domes (1 mm) to flat or umbilicated plaques (2–5 mm), which tended to coalesce. Extensive white discoloration of lungs and granulomatous pneumonia were evident by 72 hours after intra-air sac infection with *A. fumigatus* (54).

Lung lesions in experimental aerosol infection of turkey poults consisted of small white caseous nodules (approximately 1 mm in diameter) scattered throughout lung tissue (Fig. 25.3B), usually accompanied by similar sized caseous plaques on thickened air sac membranes (100, 109) (Fig. 25.3C). Occasionally, red-tinged ascites was present.

Durant and Tucker (28) observed yellowish-white nodules up to 5 × 8 mm in the lungs of wild poults reared in captivity. In an outbreak of aspergillosis in chicks, no evidence of yellowish foci was found, but the lungs were a diffuse grayish yellow (107).

In advanced cases of aspergillosis, the organism can sporulate on the surface of the caseous lesions and on the walls of the thickened air sacs (100, 109) as evidenced by visible greenish-gray mold growth.

Caseous, gelatinous, or less commonly mucopurulent exudate may be present in the syrinx in infected birds (Fig. 25.3D). Localized tracheal aspergillosis caused by *A. flavus,* described by Barton and coworkers (9), was characterized by grossly visible yellow caseous plaques adherent to the mucosal surface that sometimes occluded the lumina. The tracheal walls were reddened as well.

Lesions in brain tissue were described by Richard *et al.* (100) as white to yellow circumscribed areas (Fig. 25.3E) usually visible on the brain surface. They were present either in the cerebellum or cerebrum or less frequently in both.

Julian and Goryo (50) described ascites in chickens as a frequent sequela to pulmonary aspergillosis caused by *A. fumigatus.* Acute cor pulmonale was the suspected initiator of the vascular failure.

Mohler and Buckley (68) described lesions in a flamingo consisting of pseudomembranous bronchitis, in addition to lung nodules. Similarly, airway-centered pseudomembranous lesions were traced from bronchial lumina through parabronchi extending to the pleural surface lesions in an ostrich that succumbed to aspergillosis (4). In another ostrich case, the lungs were covered with miliary foci (49). In canaries observed by De Jong (26), there were small, whitish-yellow, crusty coatings on the tongue, palate, and aditus laryngis, and in the trachea and syrinx. Caseous foci in lungs and caseous coatings on the pleura and peritoneum were also observed. Lahaye (58) stated that *A. glaucus* may be the cause of a skin disease in pigeons, particularly in young birds, and that any part of the body may be affected with yellow scaly spots. Feathers in the affected areas were dry and easily broken.

Histopathology

In a study of acute pulmonary aspergillosis in turkey poults, granulomatous airsacculitis and pleuritis were seen as early as 24 hours after intra-air sac inoculation with *A. fumigatus* (54). Air sac membranes were thickened up to 100-fold by massive infiltrates of heterophils, multinucleate giant cells, and other leukocytes. Germinating conidia were seen in the membrane interstitium, and lymphohistiocytic perivasculitis was discernable in less severely affected areas. Granulomas had centers composed of necrotic cellular debris and heterophils with a peripheral palisade of epithelioid macrophages and aggregates of lymphocytes (Fig. 25.3F). Examination of pyogranulomas stained with Gomori's methenamine silver stain revealed large numbers of germinating conidia centrally and hyphae extending peripherally through the layer of macrophages (Fig. 25.3G). Lung lesions consisted of heterophilic and lymphohistiocytic or granulomatous pleuritis and pneumonia with edema and hemorrhage in the initial 48 hours, but had progressed to extensive effacement of parenchymal architecture by necrosis, hemorrhage, and massive infitrates of leukocytes by 72 hours. Epithelioid macrophages admixed with multinucleate giant cells were arranged in sheets. Intact and degenerate heterophils predominated in areas of necrosis. Septate hyphae were mostly localized to areas of necrosis and aggregates of multinucleate giant cells.

Nonviable *A. fumigatus* conidia produced a transient airsacculitis and pneumonia characterized by edema and infiltrates of heterophils and macrophages (55). Multinucleate giant cells were not present in these lesions, in contrast to active infections with *Aspergillus* in which both uninuclear epithelioid and multinucleate giant-cell macrophages are a prominent feature.

In a study of subacute and chronic phases of aspergillosis, the examination of lung tissues from turkey poults revealed no differences in histopathologic lesions caused by *A. fumigatus* or *A. flavus* (97). Lesions seen in the first 2 weeks of the study were characterized by focal accumulations of lymphocytes, some macrophages, and a few giant cells. Later, lesions consisted of granulomas with a central area of necrosis containing heterophils surrounded by macrophages, giant cells, lymphocytes, and some fibrous tissue. By 8 weeks postexposure, surviving poults had mature fibrous granulomatous lesions consisting of a necrotic center surrounded by giant cells and a thick layer of fibrocytes and collagen containing a few scattered heterophils. Using Gridley's fungal stain, the organisms could be seen within the necrotic areas of the lesions. In tissue sections of the well-oxygenated bronchi, bronchioles, and air sacs, the organism was sporulating asexually.

Brain lesions consisted of solitary abscesses with necrotic centers infiltrated with heterophils and surrounded by giant cells. Hyphae were seen in the central area of some lesions.

Eye lesions were characterized by edema of the pecten, which was infiltrated heavily with heterophils and mononuclear cells. Granulomas were found in the pecten. Fungal hyphae, heterophils, macrophages, and cellular debris were found in the chambers and retina of the eye. Edema and some heterophils were found in the sclera and surrounding tissues. In cases of ophthalmitis in turkeys described by Moore (69), primary involvement was in the vitreous humor and adjoining tissues. In one turkey, he observed hyphae in the center of the lens.

In tracheal lesions, occlusions consisted of fungal mycelia and pyogranulomatous exudate, although the mucosa was necrotic and infiltrated with macrophages, and fibroplasia was evident in the subadjacent tracheal wall (9).

Pathogenesis

The conidia of *A. fumigatus* are about 3 μm in diameter; once inhaled, they are deposited deep within the respiratory tract. In a study of infected chick lung tissue, Campbell demonstrated that conidia adhered to epithelial surfaces and smooth muscle cells lining the apices of interatrial septae of parabronchi shortly after their inhalation (16). The conidia swelled slightly prior to producing germ tubes, which by 25 hours had developed into septate hyphae. Presumably, this transformation occurred in both intra- and extracellular locations as conidia were found on and in host cells. Hyphae were frequently seen within the cytoplasm of giant cells with little evidence of detriment to either. Examination of infected air sacs revealed that conidia attached to the membrane surface within 1 hour and were translocated from the luminal surface to the interior of the membrane, where conidial germination was evident within 24 hours (54, 55). Necrosis of tissue was concurrent with the fulminant inflammatory response which, by 24 hours, included massive numbers of heterophils admixed with cellular debris. Proteases expressed during the vegetative growth of *A. fumigatus*, in addition to heterophil mediated lysis, may contribute significantly to the destruction of pulmonary parenchyma (46).

Conidia of *A. fumigatus* may be disseminated by the hematogenous route in poultry as has been described in humans (105). Richard and Thurston (97) isolated *A. fumigatus* from the blood of turkeys immediately after a 15-minute aerosol exposure of conidia. At this time, macrophages harvested by lung lavage contained numerous ingested conidia. This may be the route of dissemination resulting in the eye and brain lesions (100). Some experimentally exposed turkeys had a notable torticollis, and brain lesions were found at necropsy. Torticollis occurs also in naturally occurring infections of *A. fumigatus* in turkeys, geese, and chickens (120). Usually, by 24 hours postexposure the organism was cleared from the bloodstream.

Immunity

Evidence for immunity against aspergillosis in poultry is lacking; however the majority of turkeys experimentally infected with *A. fumigatus* recover from the lesions of pulmonary aspergillosis in 4–5 weeks, if not re-exposed to the agent (55, 57). Likewise, resolution of pulmonary aspergillosis has been described in Japanese quail surviving experimental infection (19). The mechanisms involved in convalescence from aspergillosis in avian species have not been described. It has been proposed that macrophages and neutrophils alone, independent of lymphocyte responses, clear *Aspergillus* from pulmonary tissues in immunocompetent mammals (108); however, immunologic memory is assumed to have a role in protecting mice against lethal challenge subsequent to low-dose challenge (111).

Vaccination of turkeys against aspergillosis has yielded only limited protection in experimental settings by decreasing mortality (99) or lessening early histopathologic lesions (94). However, vaccination did not protect against the development of grossly apparent pulmonary lesions and may have predisposed to chronic *A. fumigatus* infection, in that some vaccinates remained culture-positive and that nonvaccinated controls were culture-negative at 8 weeks post-challenge.

Natural recovery from aspergillosis does not appear to confer protection in turkeys. Convalescent turkeys remained susceptible to pulmonary aspergillosis in a study in which recovery from unilateral *A. fumigatus*-induced airsacculitis did not protect against contralateral air sac challenge (56). Likewise, passive cellular immunization failed to protect turkeys against *A. fumigatus* challenge and splenic lymphocytes did not respond to conidial antigen preparations, regardless of the previous exposure status of the donor turkey (57).

Diagnosis

Isolation and Identification of Causative Agent

Aspergillosis usually is diagnosed at postmortem examination, often based upon the observation of white caseous nodules in the lungs or air sacs of affected birds. Bronchoscopy was used, however, in observing plaques in the bronchi and trachea of an ostrich, and biopsy specimens of infected material were obtained for histologic evaluation and bacterial and fungal examination and culture (65). Although it is sometimes possible to observe fungal growth and sporulation on the caseous nodules or plaques, especially in the air sacs, confirmation should be made by cultural isolation and identification of the causative fungus. Al-

though *A. fumigatus* is the most likely agent of avian aspergillosis, other species of *Aspergillus* can cause the disease. Therefore, isolates should be identified.

Because most agents of the mycoses are ubiquitous saprophytes, diagnostic samples should be collected carefully using aseptic technique. Samples so collected can be examined microscopically by placing a small portion of the nodule in 20% KOH on a microscope slide, teasing the material apart, and covering it with a glass coverslip. Following gentle heating of the slide over a flame, the specimen can be examined for the presence of hyphae within the exudate. If the preparation is too thick, the slide should be incubated 12–24 hours in a moist chamber and re-examined. To aid in elucidating the fungus, the KOH can be mixed with ink dye (Ink blue—pp or asb, Parker Pen Co., Janesville, WI). Hyphae of *Aspergillus* stained with the ink dye appear as blue-stained, septate, dichotomously branched structures 2–8 μm in diameter with hyphal walls generally parallel (Fig. 25.4).

Aseptically obtained specimens can be plated directly onto appropriate mycologic media. Alternatively, specimens can be placed in saline solution, minced briefly in a tissue grinder, and then streaked onto the surfaces of mycologic media. Replicate plates should be incubated at both 27° and 37°C. Collected fluids can be centrifuged, and the sediment can be examined microscopically or cultured as above.

Satisfactory media for the isolation and identification of most isolates from cases of aspergillosis include Sabouraud dextrose agar, Czapek's solution agar, and potato dextrose agar. All cultures should be examined daily, and portions of fungal colonies should be transferred to fresh media.

For light microscopic examination, a small portion of the colony containing reproductive structures can be placed in a drop of suitable mounting medium (e.g., lactophenol blue) on a clear glass slide, teased apart, covered with a coverslip, and examined.

Although an indirect histochemical technique was used to diagnose aspergillosis in a lovebird (17), the major pathogens of aspergillosis can be identified based on specific characteristics of *A. fumigatus* or *A. flavus* (see "Etiology").

Serology

Serologic tests are of limited value due to the nonspecific nature of the antigens. Agar gel precipitin tests were used by Richard *et al.* (98) in comparisons of *A. fumigatus* and *A. flavus* infections in turkey poults. Although most of the *A. fumigatus*-infected poults were positive for precipitating antibodies, poults infected with *A. flavus* were not. Peden and Rhoades found that antibody responses as measured by the enzyme-linked immunosorbent assay (ELISA) and agar gel immunodiffusion test were erratic, although most poults with high antibody scores had marked lesions and low weight (78). Additionally, a direct ELISA technique has been used in turkeys with a correlation occurring between exposure level and ELISA optical density (87). Perhaps the use of serologic methods for identifying poults with aspergillosis for culling procedures would be advantageous, but presently, there is no legal or effective therapy for treating positive birds.



25.4. Hyphae of *Aspergillus fumigatus* in lesion material prepared as a wet mount in 20% KOH and ink dye. ×450.

Differential Diagnosis

The clinical signs of avian aspergillosis are nonspecific and dependent upon the organ systems involved. Pulmonary aspergillosis usually is differentiated from other avian respiratory diseases by the granulomatous lesions observed at necropsy although Linares and Wigle (62) found *Staphylococcus aureus* pneumonia in newly placed poults can appear similar. Exudative fibrinous or purulent airsacculitis and pneumonia are also frequently seen in cases of mycoplasmosis, colibacillosis, fowl cholera, and chlamydophilosis. Mycobacteriosis and other mycoses should also be considered when granulomas predominate.

Intervention Strategies

Management Procedures

Aspergillus fumigatus infection in young chicks and poults has been somewhat controlled by hatchery sanitation. Sophisticated sampling equipment and media are available to monitor air in hatcheries. Moldy litter or feed and access to musty, moldy strawstacks should be avoided to prevent outbreaks of aspergillosis. Examination of premises or materials used for feed or litter usually will reveal the source of infection.

Areas around feed hoppers and watering places are fertile areas for growth of molds. Unless a permanent yard system is used, frequent moving of feed troughs and watering places is advisable. Placing feed containers and watering fountains on screened, elevated platforms helps to prevent turkeys from picking up molds that develop in such places. Drainage is advisable for areas where water is likely to stand after rains.

Daily cleaning and disinfection of feed and water utensils will aid in eliminating infection. Spraying the ground around containers with chemical solutions may be advisable if it is impossible

to change feeding areas frequently. In outbreaks, a 1:2000 aqueous solution of copper sulfate for all drinking water may be used to aid in preventing the spread, although it should not be relied upon as a method to be used continually. Dyar *et al.* (29) reduced the mold count of contaminated litter and the mortalities of turkeys due to aspergillosis by treating the litter with nystatin and copper sulfate. A thiabendazole solution sprayed on green oak wood shaving litter was efficacious in reducing mold spore counts in the litter, and there were reductions in pulmonary lesions of aspergillosis in turkeys raised on that litter (32). Mortality due to an aspergillosis outbreak in a broiler flock was reduced within 2 days of treatment of litter with an enilconazole solution (88).

Prevention is currently the preferred means of control. This usually involves eliminating the source of the organism, such as moldy feed and litter, and treating the poultry houses and litter with antifungal compounds. In spite of precautions and preventive measures, outbreaks of aspergillosis frequently occur in some houses and at certain times of the year, particularly during the winter in closed rearing houses. Evidently, increased ventilation within poultry rearing houses reduces the airborne mycoflora (100) suggesting that this could be used as a preventive measure in controlling aspergillosis. Natural ventilation appeared to be better than forced-air ventilation. A significant effect of the type of natural ventilation design (curtain or sliding door), however, was not evident in studies evaluating turkey performance using mortality, average daily gain, feed conversion, condemnations at slaughter, or average individual bird weight as measures of production (25).

Generally, an effective means of therapy for avian aspergillosis is not available. Although certain drugs have been used for the treatment of mammalian aspergillosis, they are apparently not cost-effective for poultry. Wawrzkiewicz and Cygan (122) studied 64 strains of *Aspergillus,* 26 of *Rhizopus,* and 2 of *Mucor* from 56 lung tissue samples of poultry with aspergillosis. The most active fungistats against these *in vitro* were nystatin, amphotericin B, crystal violet, and brilliant green. Hamycin in the drinking water was reportedly successful in controlling an outbreak of aspergillosis in young chicks (7). Miconazole was used successfully in the treatment of raptors with clinical aspergillosis (33). Infections in chick embryos have been controlled by the use of amphotericin B (45) and phenylmercuric dinaphthylmethane disulfonate (41). Dimethyldithiocarbamate, injected subcutaneously, was effective against *A. fumigatus* infection in 5- and 10-week-old chickens. The drug significantly reduced the lesion size and the rate of isolation of the organism from tissues in comparisons between treated and untreated infected birds (27). Exposure of chicks to enilconazole fumigation at the time of experimental infection with *A. fumigatus* reduced morbidity and mortality (118). In another experimental aspergillosis study comparing the efficacy of azole compounds, treatment of poults by crop gavage with itraconazole was the most effective in reducing lesion scores and weight loss (80).

Vaccines are not a practical alternative, and none are commercially available. Experimental vaccine preparations have not been efficacious. In exception, Richard *et al.* (100) reported reduced mortalities by 50% in turkey poults vaccinated with a germling (germinated conidia) vaccine prepared from *A. fumigatus* and subsequently challenge-exposed to aerosols of *A. fumigatus* conidia. Viable spores of *A. fumigatus* administered to ducks provided some protection from death following challenge exposure (5).

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Candidiasis (Thrush)

Introduction

Candida species have a worldwide distribution and are part of the microflora of the healthy digestive system of humans, animals, and birds. Candidiasis is an opportunistic endogenous mycosis in that perturbance of the microflora or other debilitation of the host, rather than exposure to an external source, is the initiator of pathologic infection. Extremes of age and concurrent disease often are implicated when immunosuppression is suspected as the underlying problem. Prolonged or otherwise inappropriate antimicrobial therapy that upsets microflora ecology is likely the most common initiator of candidiasis. Birds are particularly susceptible to oral and crop candidiasis, which resembles thrush in humans.

Definition and Synonyms

Candidiasis is a mycosis caused by infection with the mycelial yeasts of the *Candida* genus, principally *C. albicans*. The organism is commensal and an opportunistic pathogen. The term thrush is applied to *Candida* infections of the upper digestive tract. Stomatitis oidica, muguet (Fr.), Soor (Ger.), moniliasis, oidiomycosis, and sour crop are other terms applied to mycotic infections of the digestive tract.

Significance

Thrush has been observed in chickens, turkeys, geese, pigeons, guinea fowl, pheasants, ruffed grouse, quail, peacocks, lories, lovebirds, finches, parrots, and parakeets (1, 18, 23). The occurrence of avian candidiasis is sporadic, but outbreaks can be costly. The first major reported outbreak resulted in mortality of up to 20% in young turkeys (5), and another report the following year described the loss of 10,000 chicks to the fungal infection (8). A recent outbreak resulted in 40% mortality in a flock of 6-week-old turkeys (18).

History

The significance of yeast-like fungi in infections of the digestive tract of humans was recognized by Langenbeck in 1859. Historical synonyms for the disease and the etiologic agent can be confusing. Moniliasis denotes infection with the genus *Monilia*, which is outdated. *Candida* has since replaced the once familiar but invalid *Monilia* in accordance with a decision reached at the Third International Microbiological Congress in 1939. *C. albicans* is the most frequently isolated etiologic agent of candidiasis, which is sometimes yet referred to as moniliasis.

Jungherr (10) stated that *Monilia albicans* is of widespread occurrence in gallinaceous birds, pathogenic to birds and also to rabbits on intravenous (IV) injection, and indistinguishable from strains isolated from human sources. He also associated *M. albicans, M. krusei,* and *Oidium pullorum* sp. n. with cases of thrush but considered *M. krusei* to be of no etiologic significance (9, 10). *Mucor* spp. and aspergilli were also found in association with some cases. Hinshaw (7) reported *M. albicans* to be found in most cases of thrush in turkeys and chickens that came to his attention. Both investigators noted that the mycotic infections were apt to be associated with unhygienic conditions.

Studies of Benham (2), Worley and Stovall (28), Martin *et al*. (16), and others indicated the complexity of etiology. Stovall (21) presented a means of improving the identification of isolates by suggesting a specific set of environmental conditions under which the biologic characteristics of the organism were constant and could be demonstrated.

Etiology

Candida albicans is the primary agent of thrush, although other *Candida* species have been isolated from both healthy and diseased birds. In a mycological survey of crops obtained from broilers, *C. albicans* comprised 95% of the isolates, and the remainder were identified as *C. ravautii*, *C. salmonicola*, *C. guilliermondii*, *C. parapsilosis*, *C. catenulata*, or *C. brumptii* (30). Only *C. albicans* and *C. parapsilosis* were associated with the cases of crop mycosis reported in the study. An outbreak of thrush in turkeys yielded isolations of *C. albicans*, *C. rugosa*, *C. famata*, *C. tropicalis*, and *C. guilliermondii* with *C. rugosa* as the sole isolate from several of the diseased crops (18).

Pathogenesis and Epidemiology

Incidence and Distribution

C. albicans is a commensal fungus readily isolated from the intestine and mucocutaneous surfaces of birds, animals, and humans. Its distribution is worldwide. Pathology due to infection is an aberration brought on by a lapse of immunologic homeostasis or shifts in the ecology of microflora colonizing the host. The condition is sometimes seen as a sequela to coccidiostat treatment.

Mycosis of the digestive tract may occur more frequently than the paucity of diagnoses in reports from diagnostic laboratories suggests. Perhaps, in many cases, it may not be of great consequence. Serious outbreaks have been reported, however, in many species of birds. Animals and humans are also affected. Gierke (5) reported an outbreak of a thrushlike disease occurring in turkeys in California. Hart (6) reported the disease in turkeys and other fowl in New South Wales. A review of the disease in turkeys and chickens in California was recorded by Mayeda (17). A mortality rate of 40% was reported in an affected turkey flock in central Italy (18). Hinshaw (7) reported thrush in 12 flocks of turkeys, with lesions similar to those noted in chickens. Blaxland and Fincham (3) studied 5 serious outbreaks in young turkeys. Their observations supported previous conclusions that moniliasis is likely to be associated with unhygienic surroundings and other debilitating conditions.

Clinical Signs

Signs are not particularly characteristic. Affected chicks show unsatisfactory growth, stunted appearance, listlessness, and roughness of feathers. When candidiasis occurs as a secondary infection, the signs of the predisposing disease may predominate the clinical picture.

Young birds are more susceptible than older birds to mycosis of the digestive tract. Thus, as infected birds grow older, they tend to overcome the infection. Jungherr (8) observed an outbreak in which losses amounted to 10,000 chicks out of 50,000 that were less than 60 days of age. He also reported that turkeys under 4 weeks of age succumbed rapidly to infection (10), but that outbreaks in birds 3 months of age resulted in a high percentage of recoveries.

Gross Lesions

Lesions occur most frequently in the crop and consist of thickening of the mucosa with whitish, raised circular or rugose formations (Fig. 25.3H [*Note*: Fig. 25.3 can be found in "Aspergillosis" earlier in this chapter]). Often, there are curdy pseudomembranous necrotic patches that are peeled easily from the eroded mucosal surface. The mouth and esophagus may be diphtheritic and eroded.

Underwood (24) described an instrument known as McCarthy's foroblique panendoscope that was used to diagnose experimental crop moniliasis. This instrument was equipped with a viewing lens and an independent light source. Birds were starved for 12 hours to empty the crop to allow a clear view of the mucosa. A normal crop appeared to be light pink, with a glistening smooth surface having numerous shallow convolutions, whereas a fungus-infected crop showed severe corrugations to mild whitish streaks, erosions or diphtheritic formations, and a deep red surrounding mucosa.

When the proventriculus is involved, it is swollen, the serosa has a glossy appearance, and the mucosa is hemorrhagic and may be covered with catarrhal or necrotic exudate. The frequent association of mycosis of the digestive tract with other debilitating conditions, such as gizzard erosions and intestinal coccidiosis, must be considered. Gizzard erosions, as such, probably are not directly related to thrush. Likewise, the thickened intestine with watery contents frequently noted in cases of thrush is probably due to coccidiosis or other protozoan infections.

Wyatt *et al*. (29) induced systemic candidiasis in 14-day-old broiler chickens with an intravenous injection of a suspension of *C. albicans* cells. Growth was severely retarded; neural disturbances were noted; and there was marked hepatic, renal, and pancreatic congestion. Miliary abscesses are produced in kidneys of rabbits injected intravenously (2).

A case of integumentary candidiasis resulted in feather loss and superficial dermatitis in 70% of a flock of 18-month-old laying chickens (14).

Microscopic Lesions

Colonization of the keratinized stratified squamous epithelium of oral, crop, and esophageal mucosa typically is limited to the stra-

tum corneum or with extensions into the stratum spinosum. The mucosal surface may be covered by a crust composed of an admixture of necrotic debris, sloughed epithelial cells, leukocytes, bacterial colonies, and the yeast and pseudohyphal forms of *Candida*. Epidermal edema and parakeratotic hyperkeratosis may be evident. Epidermitis characterized by mixed infiltrates of macrophages, lymphoctes, plasmacytes, and heterophils is commonly seen. Epidermal and superficial dermal microabcesses, submucosal or dermal edema, and interface dermatitis may be present. Submucosal or dermal colonization with attendant inflammation is a less frequent feature.

The morphology of *Candida* in tissues is fairly characteristic in that mycelial and yeast forms are all usually present in lesions. The yeastlike cells are oval and 3–6 μm in diameter. Mycelia consist of both hyphae and pseudohyphae. Pseudohyphae are composed of elongated yeastlike cells arranged in chains that appear similar to hyphae but have prominent constrictions between adjoining cells. The hyphae have parallel sides, are septate, and measure 3–5 μm in width. Periodic acid Schiff and Gomori's methenamine silver stains are helpful in visualizing the fungal morphology in tissue sections.

Periportal focal necrosis in the liver in some cases suggested toxic action upon the system. A soluble endotoxin, toxic for mice, has been isolated from *Candida albicans.* Tripathy (22) considered that vascular damage in infected turkeys may be associated with *Candida* endotoxin. Atheromatous lesions were present on the intimal surface of the abdominal aorta in more than 50% of turkeys exposed to *C. albicans;* whereas there was only a 12.5% incidence of similar lesions in uninfected controls. *Candida* isolated from infected humans have been shown to produce a metabolite similar to the *Aspergillus* gliotoxin, a member of the chemical class epipolythiodioxopiperzine, which possess immunomodulating and antiphagocytic properties (20).

Diagnosis

A clinical picture including poor hygiene or history of long-term antimicrobial therapy is supportive when *Candidiasis* is suspected. Observation of characteristic proliferative white curd-like lesions and heavy growth, predominated by yeastlike colonies, on primary cultures, serves to diagnose thrush. Because of the possibility of cultivation of *C. albicans* from apparently normal tissues, an original heavy growth is considered essential for diagnosis. Direct microscopic examination of fresh tissue samples is useful if pseudohyphae and budding yeast can be demonstrated. *Candida* cells stain Gram-positive.

Aseptically collected scrapings of mucosal lesions can be streaked onto Sabouraud's dextrose agar with 50 μg/ml chloramphenicol and 0.5 mg/ml cycloheximide, to inhibit bacterial and mold growth, respectively. Additional agar plates with chloramphenicol only should be streaked, as some *Candida* isolates are sensitive to cycloheximide. Incubation of duplicate plates at both 27°C and 37°C is recommended. Plates should be examined daily for 5 days and discarded after 1 month. On Sabouraud dextrose agar, it produces a whitish, creamy, high-convex colony after incubation for 24–48 hours at 37°C.

Microscopic morphology of young cultures consists of oval budding yeast cells about 5.5 × 3.5 μm. Older cultures show hyphae and occasionally chlamydospores, which are spherical, swollen cells with a thickened cell wall. Formation of chlamydospores is facilitated by growth on cornmeal-Tween 80 agar or other chlamydospore agar. Clusters of blastoconidia on the sides of pseudohyphae are distinguishing features as is germ-tube production in appropriate media (13).

Candida species can be identified by substrate utilization panels. In Dunham's peptone water containing 1% fermentable substance and 1% Andrade's indicator, the organism produces acid and gas in dextrose, levulose, maltose, and mannose; slight acid in galactose and sucrose; and does not use dextrin (variable according to brand), inulin, lactose, and raffinose. Gelatin stab cultures show short villous to arborescent outgrowths without liquefaction of the medium. Commercially available carbohydrate assimilation panels, such as the API 20C yeast identification system and RapID Yeast Plus System, are used in medical diagnostic laboratories (19, 26).

Treatment and Control

Because mycosis of the digestive tract is apt to be related to unhygienic, unsanitary, overcrowded conditions, they should not be allowed to exist or should be corrected. Jungherr (9) found that denatured alcohol and coal-tar derivatives were ineffective as disinfectants and suggested that iodine preparations be used. As a treatment, he recommended that following an Epsom salt flush, 1 level teaspoon of powdered bluestone ($CuSO_4$) be added to each 2 gallons of drinking water in nonmetal containers every other day for 1 week. Hinshaw (7) recommended that a 1:2000 solution of $CuSO_4$ for turkeys be used as the sole source of drinking water during the course of the outbreak. However, Underwood *et al.* (25) found $CuSO_4$ to be ineffective for treating or preventing the disease in chicks and poults with experimentally produced moniliasis. Affected birds should be segregated for protection against cannibalism. Lesions in the mouth can be treated by local application of a suitable antiseptic. Appearance of the disease in very young chicks suggests that the surface of the egg is a source of infection. Such a possibility could be removed by dipping eggs in an iodine preparation prior to incubation.

Kostin (12) found that *C. albicans* organisms mixed with poultry droppings and applied to wooden boards could be killed by exposure to 2% formaldehyde or 1% sodium hydroxide solution for 1 hour. Treatment with a 5% solution of iodine monochloride in hydrochloric acid for 3 hours was also successful in disinfection.

Nystatin treatment has been studied by Gentry *et al*. (4) and Kahn and Weisblatt (11). One group reported that a 220 mg nystatin/kg diet was effective in eliminating moniliasis in a flock of turkeys. The other group found that in experimental infections with *C. albicans* in both chickens and turkeys, crop lesion severity appeared to be significantly reduced in the group fed the lowest level of nystatin (11 mg/kg). The highest level (110 mg/kg) showed very significant protection against mycotic infection.

Yacowitz *et al*. (31) reported the successful prevention of can-

didiasis in chickens by the addition of nystatin at a minimum level of 142 mg/kg ration for 4 weeks. Kahn and Weisblatt (11) obtained similar results. Wind and Yacowitz (27) successfully treated crop mycosis with nystatin by dispersing it in drinking water at levels of 62.5–250 mg/L with sodium lauryl sulfate (7.8–25 mg/L) for 5 days.

Tripathy (22) found that the addition of chlortetracycline (500 g/ton) to a vitamin A-deficient ration had no effect on incidence or severity of crop candidiasis but increased the cells being shed in feces. Turkeys fed nystatin (100 g/ton) had a higher average weight and milder crop lesions than untreated controls.

Lin reported results of *in vitro* drug susceptibility testing of clinical isolates of *Candida* and recommended nystatin treatment be administered by water because poultry with thrush tend to show decreased feed consumption and increased water consumption (15).

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Dermatophytosis (Favus)

Dermatophytosis, dermatomycosis, ringworm, and favus are terms applied to the condition of fungal infections of skin. The term favus usually is used to denote the disease in poultry. Favus has a worldwide distribution but its occurrence is sporadic. The infection is contagious and is transmissible to humans, as is the case with the majority of dermatophytoses. The primary etiologic agent of favus is *Microsporum gallinae*.

The etiologic agent of favus was first described in 1881 as

Epidermophyton gallinae, later known as *Achorion gallinae*, then *Trichophyton gallinae*, and eventually accepted as *Microsporum gallinae*. Favus caused by *M. gallinae* has been reported in the chicken, turkey, duck, quail, and canary (1). Although a rarely encountered disease in large-scale poultry production, *M. gallinae*-induced favus may be more common in backyard, hobby, or game chicken flocks (2, 3, 4).

M. gallinae is a primary pathogen. Other than skin lesions, affected birds are typically healthy. Favus spreads gradually through a flock by direct contact, if left unchecked, and can produce ringworm lesions in their human handlers. In chickens, infection typically produces white scaly or crusty lesions on the comb and on the skin of the head and neck with loss of feathers. Microscopically, colonization is limited to the epidermis. The skin surface may appear thickened by orthokeratotic hyperkeratosis and serocellular crusts with a primary component of heterophils admixed with mycelia. Acanthosis and acantholysis with hydropic degeneration may be present. Lymphohistiocytic and heterophilic epidermitis and dermatitis are also seen. Examination of feather follicles reveals mycelial colonization of the keratinized shaft. In tissues stained with periodic acid-Schiff or Gomori's methenamine silver stains, the fungal morphology is that of branching, septate hyphae with parallel sides, 2–5 μm in diameter.

Skin scrapings placed in a drop of 10% KOH on a glass slide, which is then cover-slipped and gently heated over a flame, can be used to visualize the intact and fragmented intralesional hyphae. Scrapings can be cultured on Sabouraud's dextrose agar with 50 μg/ml chloramphenicol and 0.5 mg/ml cycloheximide and incubated at or around 27°C. Colonies of *M. gallinae* usually will develop in 1–2 weeks at 27°C, or about 4 weeks at 20°C. Initially, colonies are white and velvety and become tinged with pink as the culture ages. The colony reverse is initially yellowish and gradually changes to red. Microscopically cultures are composed of slender (2–5 μm) branching, septate hyphae bearing abundant microconidia and fewer macroconidia. Microconidia are pyriform (pear-shaped) and measure 2 × 4 μm. Macroconidia (6–8 × 15–50 μm) have thin smooth or echinulate walls, contain 4–10 cells, are blunt tipped, and have a curved and tapering base (5).

Introduction of birds with lesions of favus into existing flocks should be avoided. Other reservoirs, such as contaminated soil, may exist, but *M. gallinae* has been isolated only from infections. Birds with favus should be segregated to prevent transmission of the agent. There is no label-approved treatment for poultry, but topical application of miconazole ointment on affected areas is apparently efficacious (3). Care should be exercised when handling birds with favus to prevent zoonotic transmission. Use of examining gloves with proper disposal after use is encouraged.

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Dactylariosis

Dactylariosis is a sporadic fungal encephalitis of birds caused by the dematiaceous thermophilic fungus *Dactylaria gallopava* (1), first described in 1964 as *Diplorhinotrichum gallopavum* (3). The disease has been described in young chickens, turkey poults, and quail chicks (3, 4, 5).

Although dactylariosis does not occur frequently, when it does, a moderately large proportion of a flock may be affected with mortality approximating the morbidity rate. Ranck *et al*. (4) described an outbreak in a flock of young chickens resulting in fatal encephalitis in 200 birds from a flock of 65,000. The disease was reproduced experimentally by a spore suspension injected into the left posterior thoracic air sac, left maxillary sinus, and cerebrum. Waldrip (7) reported an outbreak in 60,000 broilers with a mortality rate of 3–5%. Blalock described an outbreak of dactylariosis in turkey poults causing 20% mortality (2). Shane (5) reported a mortality rate of 15–20% in an outbreak involving Japanese quail chicks.

Clinical signs are consistent with central nervous system pathology and include incoordination, loss of equilibrium, tremors, torticollis, paralysis, and death. Gross lesions may be confined to the brain with involvement of both the cerebellum and cerebrum. Lesions have been described as large, hardened, grayish, and circumscribed (2) or as focally extensive areas of prominent red discoloration (5). Pulmonary granulomas are seen in some cases (2).

Histologically, lesions are characterized by multifocal to coalescing areas of infiltrates of massive numbers of heterophils, macrophages, and multinucleated giant cells with central areas of necrosis. The dematiaceous hyphae of *Dactylaria* are readily apparent in hematoxylin and eosin stained tissue sections. The hyphae typically are scattered throughout the lesion in a random arrangement and are yellow to light brown, septate, irregularly branched, and 1.2–2.4 μm in diameter.

Small pieces of brain tissue containing the lesions can be ground and inoculated onto slants of Sabouraud's dextrose agar and incubated at both 24°C and 37°C. *Dactylaria gallopava*

grows well at both room temperature and 37°C; however, maximum growth of this thermophilic fungus is achieved at 45°C. Chloramphenicol (0.05 g/liter) may be used in the media to retard bacterial growth. Growth is inhibited on media containing cycloheximide (4). Within 2–5 days at 24°C or 37°C, the colonies appear on Sabouraud's dextrose agar as velvety, gray-brown colonies with a flat or wrinkled surface, and the reverse side of the colony is a deep purple-red. The red pigment diffuses into the surrounding medium, forming a halo around the colony. Microscopic examination will show light-tan-to-brown septate hyphae and oval two-celled, brownish conidia (3.2 × 9.0 μm) borne on short unbranched conidiophores.

Occurrences of dactylariosis have been associated with contaminated litter and egg incubators. Introduction of contaminated wood chip and sawdust litter was implicated in an outbreak in chickens (7). The organism apparently prefers acidic environments with moderately high temperatures.

Dactylaria gallopava occurs and grows in effluents of acid hot springs, acid thermal soils, and coal waste piles (6). Removal of contaminated litter and decontamination of incubators by fumigation is recommended when outbreaks occur.

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Sporadic Fungal Infections

Histoplasmosis

Histoplasmosis is an infectious, but not contagious, mycotic disease of humans and animals. It has been reported commonly in zoo birds, and occasionally, in chicken and turkey populations. It occurs worldwide, especially in areas in the United States bordering the Missouri, Ohio, and Mississippi rivers where the disease appears to be indigenous. *Histoplasma capsulatum* is a dimorphic fungus that occurs as an environmental mold and as a yeast in its warm-blooded hosts. Infection is acquired by inhalation of conidia produced by the mold form.

The *Histoplasma capsulatum* organism grows readily in culture media and soil as a white to brown mold that bears spores of two types: 1) spherical, minutely spiny microconidia 3–4 μm in diameter and 2) spherical, or rarely clavate, macroconidia 8–12 μm in diameter, with evenly spaced finger-like projections. The organism grows in the yeast-like phase, but with difficulty. It requires a temperature of 37°C, a medium rich in protein, preferably blood, and high levels of humidity and carbon dioxide.

The mycelial phase grows on Sabouraud's medium, dextrose agar, potato, gelatin, or bread at any temperature. Colonies appear as white to brownish mold after 2 weeks. The segmented branched hyphae are 2.5 μm wide and give rise to chlamydospores, often in chains with large round cells 20 μm in diameter.

Dodge (1) found the organism in samples from a starling roost in Italy and in soil samples from the adjacent schoolyard; a high proportion of the school children were histoplasmin-positive.

Diagnosis is based on 3 criteria: culture of the organism, histopathology, and histoplasmin sensitivity. The characteristic histopathology is histiocytic to granulomatous inflammation with intracytoplasmic narrow-based budding yeasts measuring 2–4 μm in diameter.

Mold cultures of *H. capsulatum* should be handled with disposable gloves within a biological safety cabinet and care should be exercised to disinfect equipment with phenol-based disinfectants. Plates should not be removed from the safety cabinet until sealed and their surfaces disinfected.

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Cryptococcosis

Cryptococcosis is a disease of humans and animals. In humans, it is characterized by a meningitis. Synonyms are torulosis, torula, yeast meningitis, and European blastomycosis.

Although it has not been diagnosed as a pathogen in birds and epizootics have not occurred, its importance to public health and its occurrence in bird's environments warrant discussion. The disease is distributed widely around the world, and although not of economic significance in poultry, there are many sporadic reports from zoo birds.

The fungus belongs to the imperfect yeast group under the

name of *Cryptococcus neoformans.* It reproduces by budding; cells are perfectly spherical and surrounded by a thick mucilaginous capsule. Cell diameter is 4–6 μm, and the capsule is 1–2 μm thick. It grows well within 48 hours at 30°C on glucose agar.

Bisbocci (1) isolated cryptococci from a pheasant with enterohepatitis. Chickens were experimentally infected and developed the disease. Lesions consisted of granulomas and necrotic processes in liver, intestines, lungs, and spleen.

Emmons (3) shocked the public health world by isolating *C. neoformans* from 16 of 19 premises and 63 of 111 specimens of pigeon droppings. The organism was found in the dropping sites but was not isolated from 20 pigeons examined. It appeared to grow as a saprophyte. Bishop *et al*. (2) confirmed those findings by isolating *C. neoformans* from 6 of 13 samples of pigeon nests and droppings.

Staib (5) isolated cryptococci from 28 fecal samples obtained from 201 species of birds at zoological gardens and pet shops in Germany. Twelve isolates were from canaries, one was from a wild pigeon, and the remainder were from psittacine and other birds. Fragner (4) isolated cryptococci from feces of 48 pigeons, 13 fowl, 7 pheasants, 10 house martins, 4 jackdaws and 3 chaffinches.

Infections can be diagnosed by culturing the organism. Histopathology has proved useful in diagnosis of cases in mammals. A significant feature is absence of an inflammatory reaction despite the presence of massive numbers of cryptococci in infected tissues. Mucicarmine stain is used to demonstrate the thick capsule and delineate the budding spores by imparting a deep red color to the capsule.

Prognosis is grave in cases of cryptococcal meningoencephalitis.

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Zygomycosis (Phycomycosis)

The zygomycoses primarily are caused by fungi belonging to genera *Mucor*, *Rhizopus*, *Absidia*, *Rhizomucor*, and *Mortierella* of the order Mucorales. Commonly used synonyms are mucormycosis and phycomycosis. The clinical syndromes associated with a zygomycosis are dependent upon the organ or system infected. Zygomycoses are encountered in both birds and mammals, and infection of mammals is associated with immunosuppression. In avian species, zygomycoses are uncommon. Both localized and systemic infections in birds have been reported (1). The zygomycoses are acquired from environmental sources, are not contagious, and occur in birds, animals, and humans.

Bigland (2) reported a case of systemic zygomycosis in a penguin that died following a course of clinical signs progressing from incoordination and unilateral photophobia to paralysis. A pedunculated, "orange-sized," intrathoracic, homogenous, gelatinous mass that involved ribs, vertebral column, spinal cord, and thoracic soft tissues was seen at necropsy. The posterior chamber of the enlarged globe of the right eye also contained a homogenous mass of similar texture. Nodular airsacculitis was also noted. A tentative etiologic diagnosis of *Mucor* was based on histologic and mycologic examinations.

A case of pulmonary zygomycosis in a chicken presented at necropsy with multifocal white nodules in lung parenchyma as the sole lesion. The diagnosis of zygomycosis was based upon histopathology (6). A case of zygomycotic airsacculitis, with involvement of intercostal musculature, in a duck yielded growth of *Mucor* (5).

Zygomycotic ventriculitis and proventriculitis resulting from *Rhizopus* (7) or *Mucor* infection (3) have been described in ostriches.

Zygomycoses can be diagnosed with relative assurance by histopathology. Lesions are characterized by pyogranulomatous or granulomatous inflammation usually with a pronounced component of multinucleate giant cells. Necrosis and angioinvasion are near-constant features. Granulomas typically have a necrotic center. The zygomycetes are more easily visualized with periodic acid-Schiff or Gomori's methenamine silver stain. Hyphae are relatively wide (7–20 μm) with nonparallel sides and irregular distensions, with no or few septae, and infrequent random branching.

Specific etiologic diagnosis is based upon growth and colony characteristics and microscopic morphology. Samples can be streaked on Sabouraud's dextrose agar with chloramphenicol. Cycloheximide inhibits growth. Growth is relatively rapid at 27°C, mature colonies are obtained within 4 days in most cases. Information concerning differentiation of the genera in laboratory culture is most easily obtained by consulting an illustrated laboratory manual (4).

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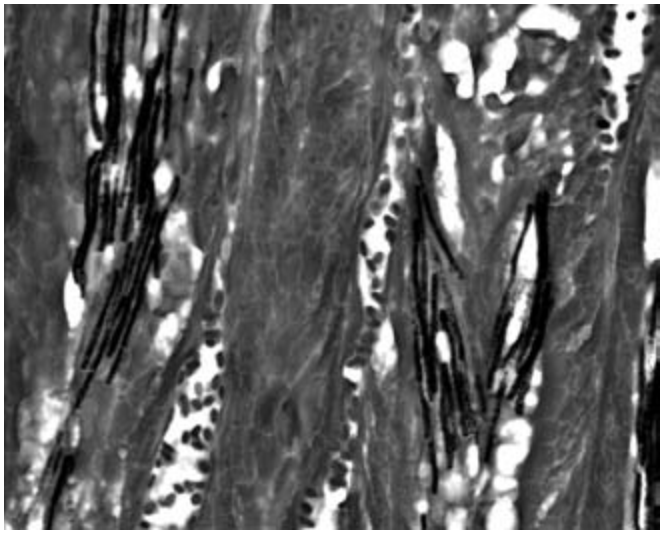
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Macrorhabdosis (Megabacteria)

Megabacteria have recently been shown to be novel anamorphic ascomycetous yeasts and not bacteria. The name *Macrorhabdus ornithogaster* has been given to the organism (8) and the disease is referred to as "avian gastric yeast infection." The organism causes a progressively debilitating, gastrointestinal disease typical of malnutrition characterized by emaciation, prostration, anorexia, cachexia, and death (2). Mortality can be as high as 90%. Characteristic large, gram-variable, PAS+ organisms are found in fecal smears and in large numbers in the proventriculi and koilin layer of the ventriculi of affected birds (Fig. 25.5). They are often arranged in parallel bundles in tissue sections and are most numerous in the isthmus between the proventriculus and ventriculus. *Macrorhabdus* needs to be differentiated from *Candida,* which is similar in size and morphology. Combined antibacterial and antifungal treatment has been used to reduce losses. Depopulation, thorough cleaning and disinfection, and leaving the premise vacant for at least 6 weeks are necessary to control the disease. Prevention in ostriches is based on isolation from pet and free-living birds, providing good husbandry and nutrition, and minimizing stress (1).

A variety of avian species are susceptible to infection with the yeast (2) and it is a serious problem among pet and aviary birds. Among domesticated birds, *Macrorhabdus* infection has been identified in chickens, turkeys, guinea fowl, quail, partridge, pigeons, ostriches, and rheas (2, 3, 4, 6, 7). Proventriculi are enlarged because of thickening of the walls and there is moderate to marked lymphoplasmacytic and heterophilic inflammation of the proventriculus and ventriculus microscopically. Numerous megabacteria, especially in areas of heterophilic inflammation, are present in the mucus, proventricular crypts, and occasionally penetrate the epithelium. Most birds with megabacteria have other concurrent diseases (4, 7).

Day-old chicks were readily infected with an isolate from a budgerigar. The organism multiplied in the proventriculus and isthmus. Efficiency of food utilization was impaired but growth was not affected (5). This study indicates the possible threat to the poultry industry posed by *Macrorhabdus.*



25.5. Large, elongated, variably stained gram-positive organisms arranged in parallel bundles in the glandular crypts of the proventriculus are diagnostic features of *Macrorhabdus ornithogaster*. Gram stain, ×400. (Dr. O. Fletcher)

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IV Parasitic Diseases

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Chapter 26

External Parasites and Poultry Pests

Nancy C. Hinkle and Leslie Hickle

Introduction to Arthropod Pests

External parasites, or "ectoparasites," are arthropods that live on or in the skin or feathers, using the host as both shelter and food source. Ectoparasites can have significant impacts on animal health and productivity. Other pests found in the environment can also have both health and economic effects. Although many avian ectoparasites exist, this chapter focuses specifically on those associated with poultry production.

Flies, beetles, mites, and other arthropod pests present concerns for commercial poultry operations. Modern high-density, confined housing systems create conditions favoring development of manure-breeding flies, beetles associated with accumulated litter, and northern fowl mites.

The three main poultry production facility types (caged-layer, broiler, and breeder houses) have their own pest problems and unique management needs. A poultry house with its stable environment, regulated temperature, high ambient humidity, and abundant food provides a nearly ideal habitat for several arthropod pests, explaining why flies and stored product beetle pests thrive in such situations. Animals maintained in close proximity to one another readily share ectoparasites, as in high-density housing.

Caged-layer houses, especially environmentally-controlled, deep-pit, or high-rise houses, are widely used for commercial egg production. Typically, they consist of two to four cage tiers on each side of an aisle, with multiple birds per cage. The deep pit below presents the greatest fly-breeding potential, because of manure accumulation under the cages.

Broiler houses are constructed as wide-span structures with litter (locally varying from rice hulls to wood shavings) covering the floor upon which the birds roam freely. The dry litter does not support maggot development, but high beetle populations may thrive under these conditions. Breeder or broiler-breeder houses are also wide-span structures with birds running free on a litter central portion surrounded on both sides by elevated slats supporting nesting boxes and hen feeders. The dead air space under the slats creates an area of high humidity, inhibiting manure drying and producing conditions conducive for fly oviposition and maggot development. Because of direct bird-to-bird contact, northern fowl mites can spread rapidly in breeder houses.

Animals have physical, physiological, and behavioral adaptations that affect their susceptibility to ectoparasites, including plumage, immunological responses, and grooming predilections. Commercial genetic (breeding) lines typically are developed and chosen for features other than resistance to ectoparasites, but host animal resistance is a characteristic that can be genetically selected and may offer future control options.

The all-in/all-out practice limits flock-to-flock transmission. Through sanitation, ectoparasite exclusion, and use of ectoparasite-free stock, poultry facilities can maintain flocks free of lice, mites, fleas, and other external parasites. Alternate hosts (such as wild birds and rodents) and contaminated fomites (egg flats, equipment, personnel, etc.) must be kept away from uninfested birds to prevent transmission. Broiler facilities seldom have ectoparasite problems because the interval from chick placement to slaughter is too short for arthropod populations to build to substantial levels.

Ectoparasites vary in their host specificity, with some (such as bird lice) living exclusively on birds. Others with intermediate host ranges (such as sticktight fleas) primarily are found on birds, but can develop successfully on mammals. Generalists (such as mosquitoes and bedbugs) are equally comfortable utilizing birds and mammals, frequently moving between the groups during their lifetimes.

Pest biology, ecology, and behavior determine the effect these arthropods have on their hosts, including infestation duration and severity. Similarly, bionomic attributes affect control strategies and determine their effectiveness.

Etiology

The three most significant arthropod pests in poultry production are flies, beetles, and mites (12).

Flies Associated with Poultry Production

Among the numerous species of muscoid fly pests on or around poultry operations, the most important develop primarily in accumulated manure and include the house fly (*Musca domestica*) and the little house fly (*Fannia canicularis*). Other flies commonly associated with poultry include blow flies (Calliphoridae), flesh flies (Sarcophagidae), dung flies (Sphaeroceridae), and fruit flies (Drosophilidae).

Classification

*House Flies (*Musca domestica*)*

House flies are gray, about 1/4 inch long, and present year-round in poultry houses, due to the suitable conditions continuously

maintained in such facilities (Fig. 26.1A). Not only are they annoying, but they can spread more than 100 disease agents to animals, including humans (20). Because they have sponging mouthparts, house flies cannot bite; however, they may play a significant role in pathogen transmission to humans and other animals. Flies carry tapeworm and nematode eggs externally and in their digestive tracts, subsequently transmitting the worms when the flies are eaten by poultry. House flies do not affect birds directly but can cause public health problems, disturb neighbors, and incite legal action by offended parties. Fly populations may create a public health nuisance around the farm and nearby communities, resulting in poor neighbor relations and threats of litigation. House flies have a potential flight range of up to 20 miles, but they are usually found within 1–2 miles of their breeding site. They reproduce in moist manure, spilled feed, and other decaying organic materials. The house fly life cycle can be completed in as little as a week at optimum conditions. House flies are active during the day, particularly when temperatures are between 25°C and 33°C. They are inactive at temperatures below 7°C.

House flies live an average of two to four weeks, with a female capable of producing up to six batches of 75 to 200 eggs at 3- to 4-day intervals. Females oviposit on moist manure, and the eggs generally hatch within 24 hours. Although development is temperature-dependent, larvae (Fig. 26.1B) can complete three instars, pupate in drier areas, and emerge as adult flies within 7–10 days in typical poultry house conditions. Adult flies tend to remain near larval development sites but may disperse several kilometers, with factors promoting dispersal poorly understood. Preferred resting sites become covered with "fly specks," consisting of regurgitated material and darker fecal spots.

House flies are implicated as carriers of rotaviruses (32), *Shigella* (22), trachoma (9), *Helicobacter pylori* (14), mycobacteria (10), *Escherichia coli* (28), *Corynebacterium pseudotuberculosis* (34), *Giardia lamblia* (8), *Vibrio cholerae* (11), and *Cryptosporidium parvum* (13).

Intervention Strategies

Management. In layer houses, if manure is rapidly dried as it is deposited, manure will form a cone-shaped mound as it accumulates, and only fresh additions at the peak will be suitable for oviposition and maggot development. Houses with scraper boards usually have drier manure rows than those without, but the effect is negated if water leaks exist.

Manure moisture is reduced by ventilation, which also maintains desirable air temperatures, removes gases such as ammonia, and provides fresh air. In environmentally controlled high-rise houses, exhaust fans located in the manure pit walls provide ventilation, drawing fresh air in through ceiling inlets, circulating it through the cage area, and pulling it out over manure in the pit. Exhaust fans located on both sides of the pit can help reduce moisture. Supplemental fans suspended above the manure rows will enhance manure drying, reducing house fly production in the pit.

Another tool that assists in managing insect populations in high-rise layer houses is in-house composting (26). Active composting is produced by incorporating a carbon source and agitating the manure to add oxygen and enhance microbial activity. Each time the manure is turned, it is heated up as microbial breakdown is stimulated, increasing the temperature and releasing additional ammonia. Core temperatures exceed the fly thermal death point, killing eggs and pupae; mobile larval stages may migrate away from the heated regions. Immobile beetle stages, likewise, are killed by this process. Constant disruption makes the habitat inhospitable for species with long life cycles, favoring colonizers.

Composting eliminates beneficial arthropods, so severe pest rebound situations may develop if composting ceases. Because turning machines cannot handle high piles, the house must be cleaned out once the pile reaches ca. 0.65 m in height. And disturbing the manure produces intense ammonia flushes, necessitating the use of appropriate protective devices.

Conscientious facility sanitation is another factor in fly control. Mortality must be removed daily and properly disposed of according to local ordinances. Spilled feed and broken eggs attract pest beetles and adult flies. Grounds maintenance, including mowing weeds and grass around houses, limits resting areas for adult flies and permits optimum airflow through the fans.

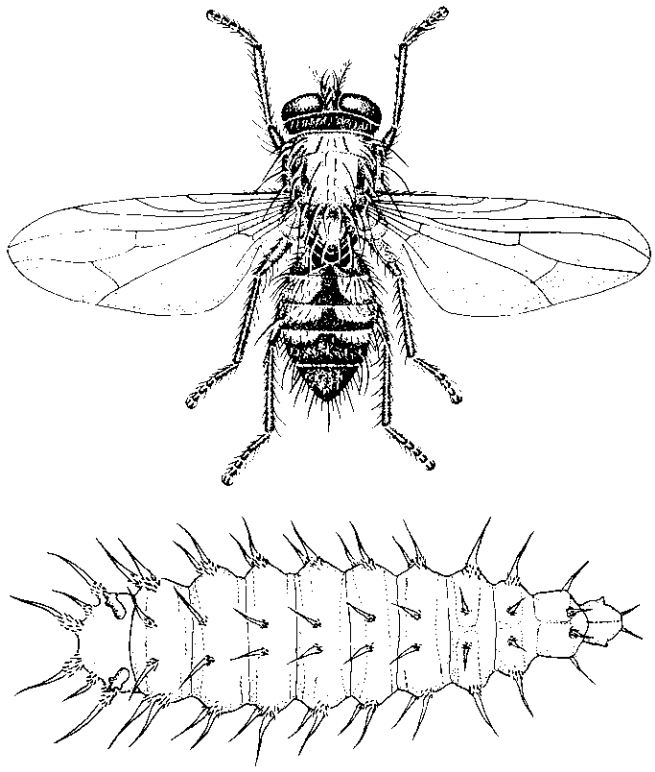
To minimize fly numbers while sustaining beneficial predator and parasitoid populations, manure management must be a year-round priority. Conserving manure and allowing it to accumulate over long intervals promotes beneficial arthropod populations. Manure produced immediately following cleanout dries slowly and has low beneficial constituents, making it ideal for fly breeding (16). Typically, severe fly outbreaks occur 3 to 6 weeks following cleanout, unless manure is removed only during cooler months when fly activity is minimal.

Chemical Treatment. Judicious use of insecticides against house flies is one component of a total house fly suppression program but should only be used as an adjunct to nonchemical strategies such as manure and moisture management. Residual insecticides applied to fly resting surfaces such as walls and overhangs or fly baits targeted at sites where flies congregate will assist in lowering adult fly numbers. Baits are ineffective when flies have access to alternative food.

*Little House Fly (*Fannia*)*

The little house fly (*Fannia*) is the predominant fly in California open-style layer houses during the winter. The little house fly resembles the house fly but is only two-thirds the size (ca. 0.4 cm) and has three brown stripes on the thoracic dorsum. At rest, *Fannia* hold their wings over the back, creating a narrower V-shape to the wing outline (Fig 26.2).

Its developmental cycle is similar to but longer than that of the house fly. Eggs are deposited on decaying organic material, especially mammal and avian excrement. Larvae hatch from the eggs in ca. 48 hours and feed in the manure, requiring eight days or longer to complete development to the pupal stage. Lesser house fly larvae are flat, brown, and spiny, a distinctive appearance retained by the pupa. Typically, egg to adult interval is two to three weeks, with the pupal period requiring almost a week. Although house flies are warm weather pests, developing optimally in summer conditions, *Fannia* develop poorly at temperatures above



26.2. *Fannia* spp. Adult and spinose larva. (Coop Extention, University of California).

27–30°C, so populations often rise in early spring, decline in midsummer, then peak again in late fall.

Male *Fannia* exhibit a distinctive lekking behavior, hovering ca. 1.7 m off the ground within poultry houses or outside in wind-protected areas, where they wait for passing females. This places them at head-level with humans, making them particularly noticeable and annoying. Strong air currents tend to disperse these male aggregations.

Little house flies are less likely to enter homes than are house flies; instead they tend to congregate in sunlit outdoor areas such as patios, entryways, and garages. They seldom land on human foods and are not considered a significant carrier of human pathogens. However, the males' habit of hovering at face height makes them annoying, although they readily move out of the way when approached.

Larval *Fannia* are adapted to utilize a wide moisture range in their developmental sites, making them particularly difficult to control through manure management (25). Oviposition and larval development frequently occur in animal wastes, but various moist organic materials can serve as suitable substrates.

Intervention Strategies

Eliminating breeding sites is the preferred method of controlling *Fannia*. Manure accumulations and other decaying organic matter provide ideal developmental sites. *Fannia* are not attracted to the same fly baits or traps that collect house flies. Placing fans in areas where male *Fannia* tend to congregate can disperse their swarms, because the increased air movement makes the site less attractive to them.

Other Pest Flies

Flies in the family Calliphoridae (blow flies, green or blue bottle flies) primarily develop in carrion and can become problems where poultry carcasses or broken eggs accumulate. Larval habitats include bird carcasses, broken eggs, dog feces, and other garbage. Adult blow flies are metallic blue, green, or black and are 1/4 to 1/2 inch long.

Small dung flies of the family Sphaeroceridae are common throughout the world, and species such as *Coproica hirtula* can be especially numerous in the weeks immediately following cleanout. They are very small, blackish or brownish flies that breed in manure and other decaying materials, often occurring in large numbers in poultry manure. Being colonizers, they frequently are among the first insects to arrive in new manure, allowing them to exploit the habitat before competitors move in. Adult sphaerocerids predominate where manure moisture is highest, typically the peak of the manure cone, but forage over a broad range of manure moisture conditions. Generally, they are not a nuisance on the farm or in nearby communities because of their fidelity to the manure ecosystem. Because they can serve as an alternative food source for beneficials such as hister beetles, control efforts are discouraged.

Fruit Fly, Drosophila melanogaster

Fruit flies are common around rotten or fermented food. They are 1/8 inch long, gray or brown, and usually have red eyes. Fruit flies lay their eggs on the surface of rotting organic materials, such as wet feed, manure, or broken eggs. The most common breeding sites are on dropping boards, or in belt houses where belts are run less than once a week. Adults are also common in egg rooms, offices, and other cool, shady areas. Fruit flies are annoying and can transmit bacteria or other disease organisms. Fly populations are highest in the winter months and early spring but decline by summer. Fruit flies are weak fliers and seem to "swarm" when workers walk through poultry facilities. *Drosophila repleta* is a pest in caged layer and breeder houses where wet feed provides suitable larval habitat, accumulating on slats, dropping boards, or other difficult to clean and treat locations (15).

Economic Significance

Although fruit flies are not known to cause direct production losses in poultry, except for their role in avian pathogen transmission, their primary economic impact is through annoyance of people on and near the poultry operation. Flies regurgitate and defecate on resting surfaces, causing unsanitary and unsightly specks on eggs, facilities, etc. Flies irritate workers and disperse to surrounding areas where the presence of flies may constitute a violation of health ordinances and create liability issues. Producers may be compelled to institute costly corrective actions or close the operation in severe cases. Because male *Fannia canicularis* assume aerial patrolling positions hovering at eye level in close proximity to humans, they are particularly visible and objectionable.

Biological Control Organisms

The black dump fly (*Hydrotaea aenescens*) is also common in poultry manure, with larvae frequently being mistaken for those of house flies and other pests. *Hydrotaea* eggs are laid in manure and hatch within 24 hours. The larvae are facultative predators, feeding both on manure and larvae of other flies. They require two weeks or more (14 to 45 days) for development to the adult stage (17). All stages are found throughout the year under suitable conditions in poultry houses. Black dump flies are 1/4 inch long, shiny, and a bronze-black color. Adults stay on the food source at night, unlike house flies and little house flies. Females lay their eggs on dead birds, spoiled feed, or very wet manure. Adult black dump flies prefer the darker areas of poultry houses and will congregate in manure pits. Black dump fly larvae are biological control agents of house fly maggots and can be mass propagated on poultry premises for inundative releases in infested houses. However, they are not entirely beneficial because populations can explode and move to neighboring homes and businesses. Like house flies, they leave vomit/fecal spots on eggs and equipment.

The soldier fly, *Hermetia illucens*, can serve as a biocontrol agent of other, more serious fly pests, but may be considered a pest itself. They are more common in high-rise, deep pit, caged-layer houses. Soldier flies are bluish black and 3/4 inch long, with large eyes and long antennae that project forward from the head. Females choose to lay their eggs in drier manure. Soldier fly larvae are large and churn manure as they develop, making the environment less hospitable to house fly maggots. They also inhibit house fly oviposition. Larvae will also feed on dead birds. Soldier fly adults are weak fliers and spend their time basking in sunny areas on structures or vegetation.

Natural biological control organisms include parasitic wasps (*Muscidifurax* and *Spalangia*), which attack fly pupae, as well as predatory mites (*Macrocheles muscaedomesticae*) and hister beetles (*Carcinops pumilio*), which feed on fly eggs and larvae (4). Manure that ages and dries supports fewer flies, due in part to the complex assemblage of natural enemies that colonize it over time (21). Because flies colonize and reproduce more rapidly than their natural enemies, flies tend to proliferate immediately after a manure cleanout. Although producers may collect extant predators/parasites for release and re-establishment following cleanout, this practice may perpetuate disease agents, as well (19).

Intervention by Management or Insecticides

Direct application of larvicides to manure is discouraged due to their deleterious effects on natural enemies of flies. Predatory and parasitic insects and mites in the manure can kill most fly immatures before they become adults.

Manure management and other forms of cultural control are critical to fly suppression, determining the type and effectiveness of other fly control methods, including chemical and biological components. For instance, frequent clean-out coupled with thin-bed manure drying may work seasonally in some areas, but it also can increase odor and dust problems.

Manure can accumulate for several months in open-sided single-story housing, or for a couple of years in two-story, deep-pit housing where manure falls into a separate pit level below the upper story that houses the hens. In these situations, manure must be effectively dried to make it less suitable for fly oviposition and larval development, while encouraging the activity of natural enemies.

Manure conditions suitable for fly development include fairly moist but not liquefied consistency, with house flies abundant in 65% to 80% moisture manure, but rare if moisture levels drop below 60% (30).

Beetles

Two beetle species associated with poultry litter and manure accumulations can cause structural damage to poultry housing, serve as potential disease reservoirs, and create community problems by migrating to nearby homes at cleanout.

*Darkling Beetles (*Alphitobius diaperinus*)*

The most significant arthropod pest in broiler production worldwide is the darkling beetle, *Alphitobius diaperinus* (Fig. 26.1C), whose larval stage is known as the lesser mealworm (Fig. 26.1D). These beetles are omnivorous, feeding on spilled feed, bird droppings, dead birds, and other components of the litter environment, as well as being cannibalistic.

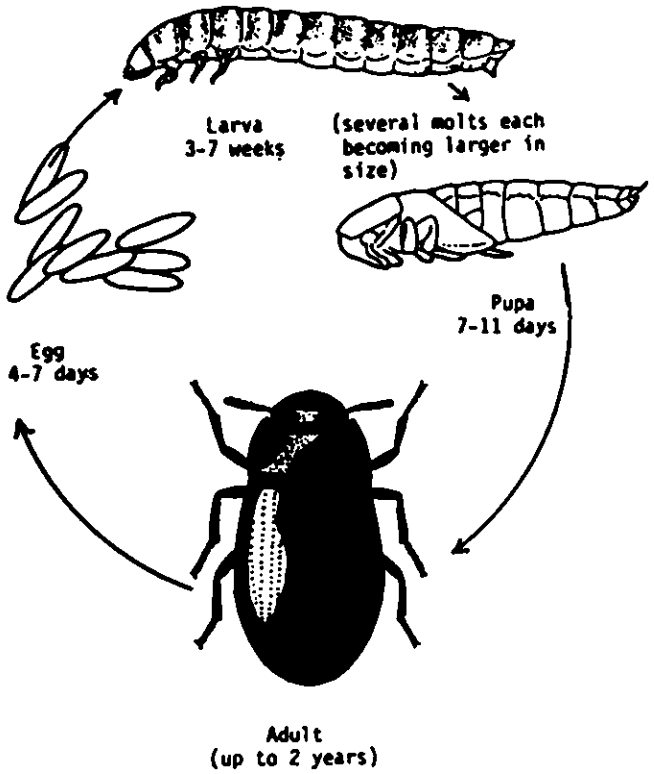
The poultry house provides an ideal environment for darkling beetles, including a temperate climate, plentiful food, a protected habitat in the litter, and relatively high humidity. There are no known predators or pathogens, but cannibalism helps regulate populations under extreme crowding.

Larvae concentrate in protected areas under feed lines, feeder trays, etc. Newly hatched larvae are less than 1 mm long, but feed and molt 6 to 11 times, reaching ca. 18 mm before pupating. The final instar larva (prepupa) is the migratory, nonfeeding stage that seeks sheltering crevices in which to pupate, hollowing out a crypt in which to conceal itself while transforming from the larval to pupal stage (Fig. 26.3). Within 3 to 13 days, the adult emerges. Adult darkling beetles are ca. 1/4 inch long, dark brown or black, with subtle striations on the wing covers. Typical adult lifespan is 3 months to 1 year. In the laboratory, darkling beetles have been shown to live over 700 days (27), with females averaging over 800 eggs in their lifetime. Generally, large numbers of beetles are not observed in houses until manure has accumulated for 5 or 6 months.

Alphitobius beetles have been shown to harbor several poultry pathogens including *Escherichia coli*, *Salmonella typhimurium* (5), *Campylobacter jejuni* (29, 31), and the viral agents of fowl pox, avian leukosis, Marek's disease, Newcastle disease, and avian influenza. Additionally, they serve as intermediate hosts of several helminths (3).

These beetles cause structural damage when prepupae tunnel into insulation and structural materials to pupate, and large populations may present problems at clean-out because of migration from fields into nearby residential areas. While beetles are capable of flying up to a mile, they typically move by crawling.

Tunneling activity of lesser mealworm larvae may aerate and



26.3. Life cycle of the darkling beetle.

dry the accumulated manure, permitting penetration by predaceous beetles and mites.

Darkling beetles cannot survive prolonged exposure to freezing temperatures, so houses in cold climates can utilize between-flock chilling to reduce beetle numbers. Pupae in insulated protected areas where micro-habitat temperatures do not drop below 0°C (clay floors, expanded foam insulation, etc.) will survive to perpetuate the infestation.

*Hide Beetle (*Dermestes maculatus*) and Larder Beetle (*D. lardarius*)*

Hide beetles are scavengers, attacking carcasses and feathers, as well as causing structural damage to facilities as prepupae tunnel in preparation for pupation. Mature larvae stay in poultry litter or bore into structures (wood, paneling, dry wall, or insulation) to pupate. As a result, "honeycombing" and structural weakness may occur. Adults are 1/3 inch long—slightly larger than darkling beetles. Hide beetles are dark brown with white ventral pilosity. Larvae are thickly covered with long hairs and reach a length of almost 18 mm before pupating. They are scavengers and will feed on dead birds, skins, hides, feathers, dead insects, or broken eggs. Because they are capable of digesting keratin, hide beetles assist in the breakdown and recycling of shed feathers. Females lay their eggs on manure and litter in poultry facilities. The hide beetle life cycle is completed in four to nine weeks, with adults living two to three months.

Beetle Suppression

Darkling beetles are more prevalent in broiler houses, while both darkling beetles and hide beetles frequently occur in layer facilities. Beetle control can be particularly challenging in broiler houses, because most pesticides cannot be applied when birds are present, limiting control activities to intervals between flocks. After a poultry house becomes infested with darkling beetles, they migrate throughout the house, making control difficult. Thoroughly cleaning the house and treating with an approved insecticide typically will suppress the population for a short time. Migration may be reduced by using physical barriers to prevent larvae and adults from climbing walls and posts.

Broiler production practices limit beetle control options. When complete clean-out is delayed for prolonged intervals, deep litter becomes a haven for the beetles, providing food, warmth, and a protected environment. If litter or cake is taken from the house and piled nearby, beetles will migrate back into the houses, so cleanout should be followed by immediate removal of litter from the premises.

At cleanout time, large numbers of beetles may disperse into the community. A litter pile can be solarized by covering it with a tarp that is sealed at the base to prevent pest emigration. Heat built up within the pile kills beetles; however, anaerobic conditions produce noxious odors and create condensate.

Applying dusts and sprays to litter and manure can be effective, but such tactics can destroy existing fly biological control agents. Many of the approved insecticides rapidly degrade under the high pH conditions found in poultry litter, so do not maintain their effectiveness very long. Additionally, the larva is able to burrow into the built-up litter, evading insecticides. In-house pesticide applications can help to reduce beetle numbers if applied at vulnerable points. The most effective strategy targets insecticide application following flock removal, immediately after the litter is decaked. Once birds exit the house, beetles migrate, so timing is crucial. In cool weather, leaving the house heated for a few days will stimulate beetle activity, maximizing their exposure to the pesticide residue. Another opportune treatment time is about the third week of the flock cycle, targeting spot treatments to the area directly beneath feeders before beetles and larvae that are concentrated under feeders begin to disperse. The second treatment opportunity is limited to insecticides that can be used while birds are present, so attention to label restrictions is critical.

Mites

The northern fowl mite, *Ornithonyssus sylviarum* (Family: Macronyssidae) (Fig. 26.4) is the most important mite on caged-layers, breeders, range turkeys, and pheasants. Northern fowl mites feed on blood and can cause anemia, itching, irritation, and reduce egg production by 10–15%. Large northern fowl mite populations can cause direct economic losses by affecting bird health and productivity (7). Heavy infestations (> 50,000 mites) can drain up to 6% of a bird's blood daily, lowering weight gains and, in male birds, reducing seminal fluid volume. Mites also annoy egg handlers and other personnel. The infestation originates on the vent and then moves to the tail, back, and legs of fe-



26.4. Northern fowl mite (*Ornithonyssus sylviarum).* (J. P. Owen)



26.5. Two levels of feather blackening and spoiling by northern fowl mite. (Matthysse)

males; the mites are more scattered on male birds. Infested poultry have feathers soiled with mite eggs, cast skins, dried blood, and excrement. The most obvious signs that a bird is infested with northern fowl mites are black feathers and scabs in the vent area (Fig. 26.5). Northern fowl mites flourish in colder weather and become well established in large numbers after poultry reach sexual maturity, being more common on four- to ten-month-old birds. Older birds apparently develop resistance to mites, seldom supporting the high mite numbers experienced by younger birds. The life cycle consists of egg, larva, two nymphal stages, and adult, and can be completed in as little as one week. All life stages occur on the host, but mites can survive for a few weeks off the avian host and readily move along cage wires to infest other birds (24). Rodents and wild birds are reservoirs for the mites and help spread them to poultry.

Northern Fowl Mite Management

Northern fowl mite control in caged layer operations depends on efforts to prevent infestation and to apply an acaricide when infestation occurs. Mite-free birds should be used to stock clean houses, to prevent northern fowl mite infestations. Because mites can be introduced on personnel, equipment, and wild animals (e.g. rodents and birds), flocks should be regularly inspected for mites. Using a bright light the inspector should part the feathers around the vent and look for mites, mite debris (eggs, shed skins, and feces), and scabbing. Caught early, an infestation can be forestalled by treating birds in proximity to the infested birds to limit mites from radiating outward.

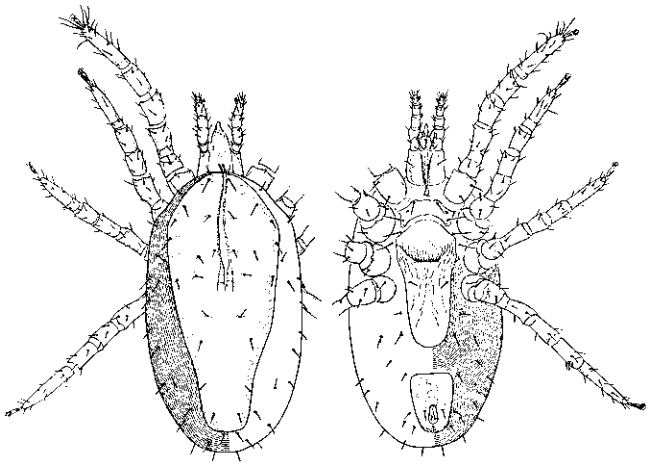
Effective chemical control in caged layer operations requires direct acaricide application to the vent region with sufficient sprayer pressure to penetrate the feathers and deliver the pesticide to the skin. To reach the vent, the spray wand (delivering spray at 100 to 125 psi) must deliver the spray upward from beneath the cages. Ready-to-use dust formulations are available for mite control and can be applied to caged layers using a power blower. In broiler-breeder operations and other situations where birds are not confined to cages treatment is more difficult, necessitating individual application.

Typically, mite population buildups are greatest in young flocks, while older birds seldom support numbers necessitating treatment. Mite populations are generally highest in cool months, and decline in summer.

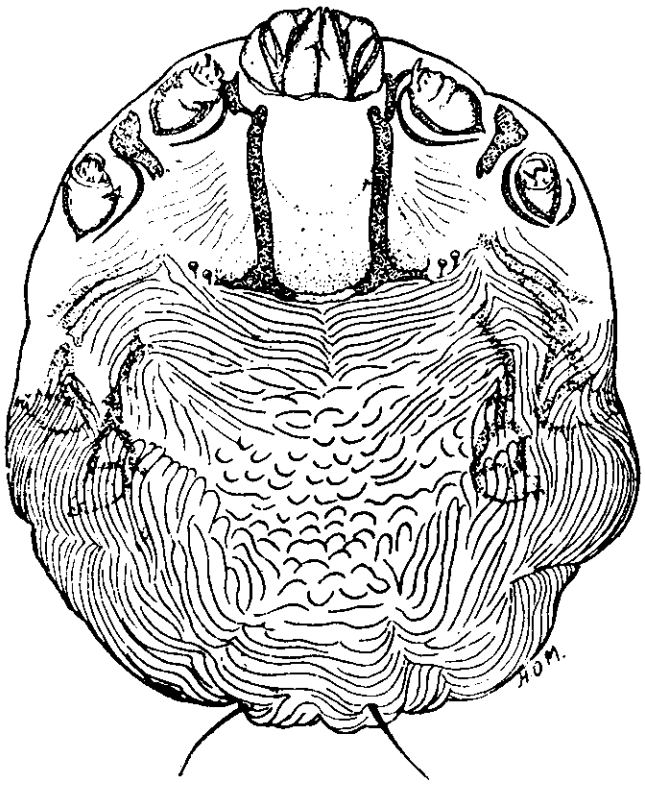
On the farm, personnel should always move from mite-free areas to mite-infested flocks, to avoid transferring mites to uninfested animals. Because mites can survive on equipment, clothing, and other materials, each house should have duplicated equipment, to prevent transporting mites as items are moved among houses.

Other Mites

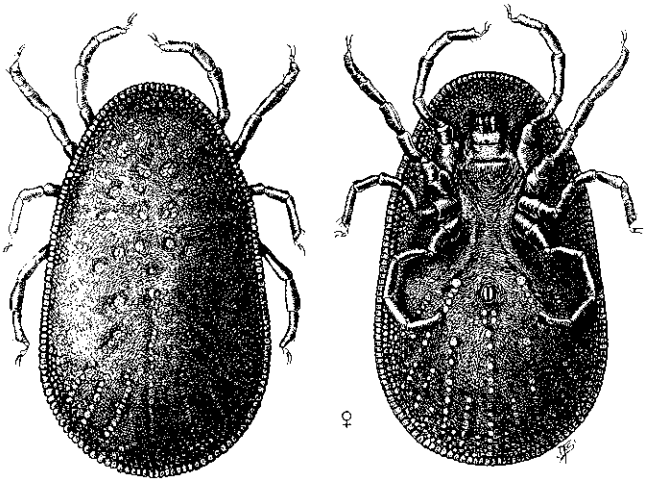
The chicken mite, *Dermanyssus gallinae* (Fig. 26.6), also known as the red mite or roost mite, is an occasional problem in turkey breeder facilities and grow-out houses. These parasites are visible to the naked eye and complete their life cycle in as little as seven to ten days. Chicken mites have been known to spread fowl cholera. They are transmitted to poultry via wild birds or rodents. Unlike the northern fowl mite, chicken mites spend only part of their time on the poultry host, moving onto the birds at night to feed, and hiding in crevices during the day. They can survive off their host for up to a month and will infest poultry workers or



26.6. *Dermanyssus gallinae,* chicken mite. (Baker)



26.7. *Knemidocoptes mutans,* scaly leg mite. (Soulsby)



26.8. *Argas persicus*, fowl tick. Dosal view on left, ventral on right. (USDA)

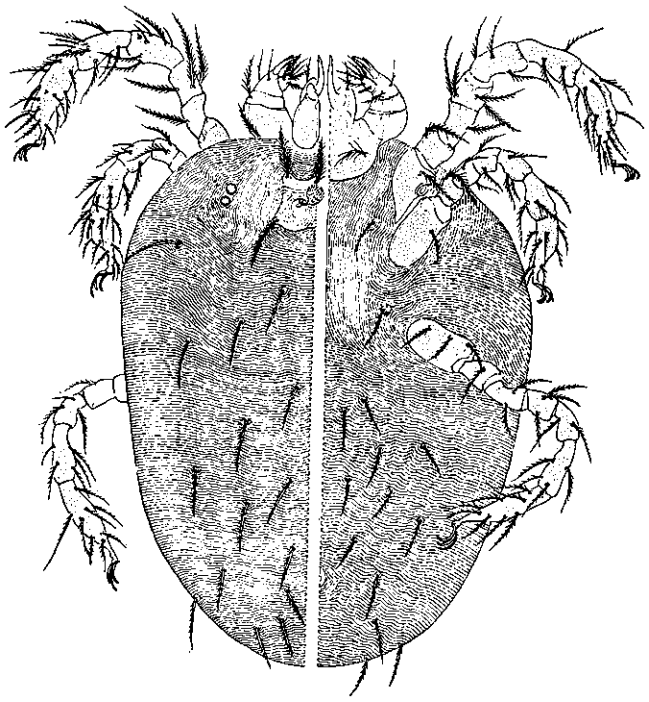
nearby facilities when infestation levels are high. In high numbers, chicken mites cause a reduction in weight gain and egg production.

The chicken mite life cycle consists of egg, larva, protonymph, deutonymph, and adult. Within 12 to 24 hours of her first blood meal, a fertilized female can lay a batch of three to seven eggs in a crevice or under debris in the poultry house. Eggs hatch within two or three days and the larva, which does not feed, molts in one to two days to the protonymph, which feeds on blood. A few days later, it molts to the deutonymph, which does not feed but molts to the adult stage after one or two days. Under optimal conditions, a life cycle (egg to egg-laying adult) requires only 7 to 9 days. However, both feeding nymphs and adults can survive several weeks without blood meals, greatly increasing the life cycle duration.

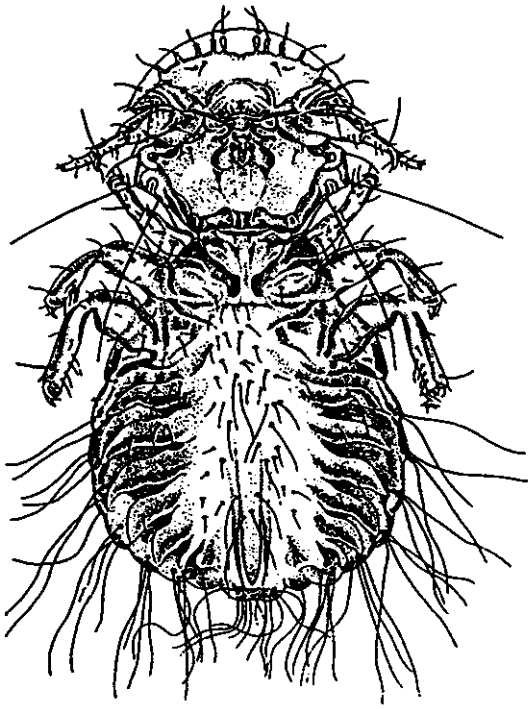
Scaly leg mites, *Knemidocoptes mutans* (Fig. 26.7), burrow under the leg scales of chickens, turkeys, pheasants, and other birds. Their presence stimulates host epithelial proliferation, resulting in hypertrophy and cornification. The legs become thickened and distorted; affected birds may be crippled if the infestation is severe. Scaly leg mites are found on wild birds, which can introduce infestations into domestic flocks. These mites complete their life cycle within 10 to 14 days.

Depluming mites, *Neocnemidocoptes laevis* var. *gallinae*, are similar to, but smaller than, the scaly leg mite. They are present throughout the United States on chickens, geese, and pheasants. Depluming mites burrow into the skin at the base of feathers on the back, wings, vent, breast, and thighs, causing intense itching and feather pulling. They are more prevalent in spring and summer, with very low numbers in autumn. The life cycle takes between 10 and 14 days to complete. Depluming mites are more common in noncommercial flocks.

Fowl ticks, *Argas persicus* (Fig. 26.8), or blue bugs, are rare pests of commercial poultry. They are light red to dark brown, 6–9 mm long as adults, and have wrinkled skin. Female fowl ticks lay their eggs in cracks and crevices near the flock. Fowl ticks are active at night, moving to the birds and taking a blood meal, causing roosting birds to act flustered. During the day these ticks secrete themselves in protected hiding places nearby. The life cycle can be completed in as little as one month. All life stages of fowl tick feed on blood, but they can live for up to a year without feeding. Ticks can transmit various bacterial and rickettsial diseases.



26.9. *Neoschongastia americana* larva, chigger of chickens. (Baker)



26.10. *Menopon gallinae*, shaft louse. (Kriner)

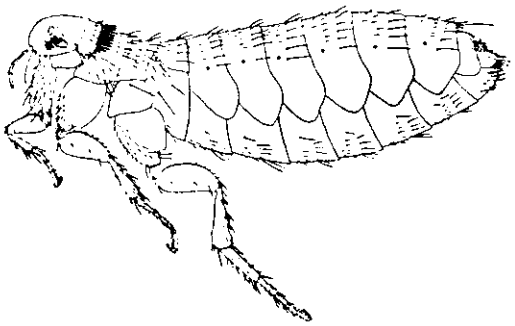
Chiggers, *Trombicula splendens*, *Neotrombicula alfreddugesi*, and *Neoschongastia americana* (Fig. 26.9), also known as harvest mites and red bugs, are bright red and less than 1 mm long. Birds on range may be exposed to chiggers, but housed animals are not at risk. *Trombicula splendens* feed on all kinds of animals and are most common in damp habitats. Common chiggers are prevalent in transition areas between forests and grasslands, swamp margins, berry patches, and thickets. They occur on a variety of animal hosts, as well. The chigger life cycle takes between 50 and 55 days, but the duration depends on habitat, temperature, humidity, and food quality. Larvae do not burrow or suck blood. Instead, they inject an enzyme into the animal host that causes irritation and a raised wheal. Bird carcass quality is greatly reduced due to raised, red chigger bite welts. Nymphs and adults are predaceous on insect eggs or immature arthropods. Turkeys are affected more often than chickens. Young poultry may refuse to eat and eventually die. *N. americana* is prevalent in the southern states of the United States in dry areas with hard, rocky soils. The population peaks in June, decreases in late summer, may increase in fall, and declines in the winter. *N. americana* infests chickens, turkeys, and wild birds. The chiggers feed in clusters on the thighs, breast, underside of the wings, and around the vent. Scabby lesions result and may take weeks to heal, causing carcass devaluation.

Lice

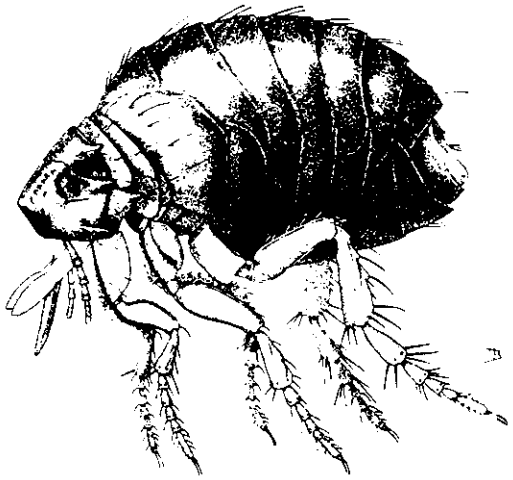
The chicken body louse, *Menacanthus stramineus* (Figs. 26.1E, F), and the shaft louse, *Menopon gallinae* (Fig. 26.10), are ectoparasites of longer-lived chickens in layer and breeder flocks. These are chewing lice, feeding on dry skin and feathers, with the entire life cycle (egg, nymph, and adult) occurring on the host. Female lice glue their eggs (nits) to host feathers. These eggs hatch into nymphs in about four to seven days. Nymphs have the same feeding habits as adults and resemble adults, except that they are smaller in size. Nymphs undergo several molts, reaching adulthood in about three to four weeks (6). The shaft louse is also known as the feather louse. These lice have chewing mouthparts and do not pierce the skin, but may feed on the blood in young quill feathers, biting into the shaft. The feeding habits of poultry lice actually make the avian host inhospitable to northern fowl mites, limiting mite infestation of lousy birds. Poultry lice are yellowish and approximately 1/16 inch long. They cause irritation, which leads to appetite loss and increased disease susceptibility. They are not specific to poultry, but are found on several wild bird species. Symptoms include red, scabby, irritated skin and reduced egg production (33). Pesticide resistance is not known in bird lice and recommended insecticides are typically quite effective in eliminating infestations, though entire flocks must be treated to avoid reinfestation.

Fleas

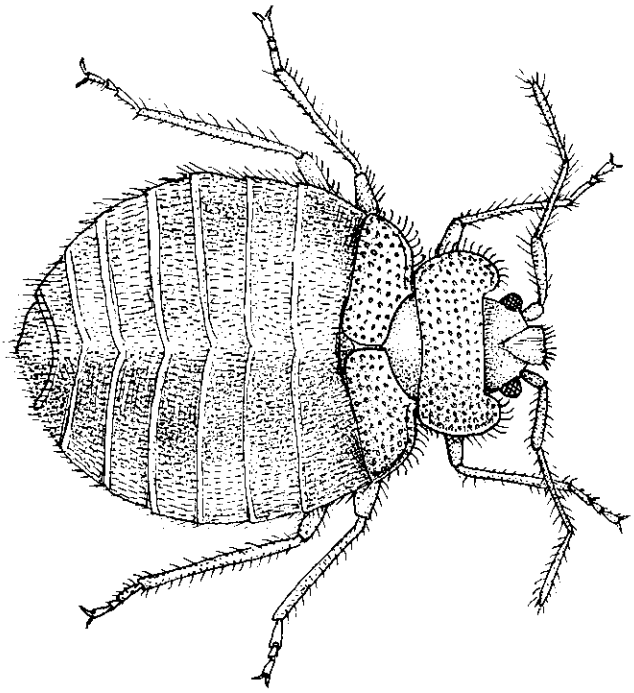
Fleas are rare in poultry facilities, but when they do occur, they are more common in breeder and grow-out houses. The European chicken flea, *Ceratophyllus gallinae*, is a nest flea that is fairly common in poultry facilities and in wild birds' nests, having been found infesting several dozen avian species (Fig. 26.11). *C. gal-*



26.11. *Ceratophyllus gallinae*, European chicken flea. (Reis and Nobrega)



26.12. *Echidnophaga gallinacea*, sticktight flea. (USDA)



26.13. *Cimex lectularius*, common bedbug. (USDA)

linae larvae develop in the nest material and feed on detritus and undigested blood excreted by the parents (23). Adult fleas leave the nest shortly after host fledging, often being carried away on the nestlings themselves (18). Flea larvae remaining in the deserted nests complete their larval development within a few days. The third larval instar spins a cocoon, pupates, and molts to the adult stage. Most of the imagoes remain quiescent in their cocoons until the next spring (18). Optimal temperature and humidity conditions for *C. gallinae* developmental stages are unknown, but larvae are known to tolerate a broad range of humidity (2), and high relative humidity seems beneficial to the survival of overwintering pharate adults in cocoons (18). Emergence from the cocoon is triggered by the spring rise in temperatures and mechanical disturbances (18).

The sticktight flea, *Echidnophaga gallinacea*, is also known as the southern chicken flea (Fig. 26.12). Adult females are permanently affixed to the host via their mouthparts, which are tightly embedded in the skin. Females attach themselves and lay their eggs on the face and wattles of poultry. Sticktight fleas may also attack mammals, especially dogs and cats, in close proximity to the infested flock. The life cycle of the sticktight flea lasts between two weeks and eight months. Young fowl may die, while older birds exhibit reduced egg production and anemia. Other symptoms include reduced growth, blood loss, and skin irritation. Sticktight fleas are more common in late spring and early summer.

Bed Bug, *Cimex lectularius*

Bed bugs are flat, 1/5 inch long, wingless, and bloodsucking (Fig. 26.13). Bed bugs feed at night, hide during the day, and lay their eggs in cracks of walls and other dark crevices. They can survive one to five months without feeding. The bed bug life cycle is completed in one to four months. Bed bugs leave fecal spots on walls, roosts, and eggs. Bed bugs may move from poultry houses into homes, infesting the human living environment. They are not known to transmit any pathogens, either mammalian or avian.

Control of Arthropod Pests

Historically, poultry pest management relied almost exclusively on pesticides to maintain pest populations below economic injury levels or nuisance thresholds. Because these thresholds were not well defined, control efforts typically were initiated when pests were observed, either on the birds or in the poultry house. Extensive or improper pesticide use results in destruction of biological control agents and pesticide resistance development in pests. It can also create harmful and illegal residues in meat and

eggs, as well as increasing human exposure and contaminating the environment. Pesticide resistance and elimination of biological control organisms result in bigger pest populations, increased pesticide use, and higher control costs.

Integrated pest management (IPM) programs for poultry combine cultural, physical, biological, and chemical control tactics (1). The first step in any IPM program is pest identification, including determining whether a problem exists, and its damage potential. For instance, mites are very common on manure in poultry houses. These are not pest mites, but beneficial mites that prey on flies and other pests. So presence of an arthropod does not constitute a problem. Once a problem has been identified, appropriate management techniques should be considered. Producers incorporate multiple pest management strategies into their production practices. Manure management, moisture control, sanitation, and pesticide applications are integrated with sound flock management practices to keep pest populations below economically damaging or nuisance levels. Successful IPM programs yield better community relations, improved flock performance, reduced structural damage, and lower control costs. Arthropod pest management must be implemented in concert and compatibly with other management considerations such as flock health, nutrition, facilities design, and production economics.

Cultural and Physical Control

While adult flies are considered the pestiferous stage, the larval stage should be the prime target for suppression. Fly control is dependent on manure and moisture management. Dry manure (50% moisture or less) is not suitable for fly oviposition or larval development, but simultaneously provides a desirable habitat for beneficial predators and parasitoids. Moist poultry manure is highly attractive to adult flies, emanating volatiles that lure in gravid females and stimulate oviposition. Fresh poultry manure at ca. 75 to 80% moisture also provides ideal conditions for larval development. Maggots can exploit manure having moisture levels between 50 and 85%. Saturation of manure from leaking waterers, condensation from noninsulated overhead water lines, improper ventilation, and seepage from outdoors can reconstitute previously dried manure, leading to maggot concentrations.

Biological Control

Several beneficial insects and mites are associated with poultry manure, including predaceous mites, hister beetles, and parasitoids. Compatible cultural and physical control practices encourage biological control by fostering retention of beneficial predators and parasitoids that can help suppress house fly populations.

A common predaceous beetle found in poultry manure is *Carcinops pumilio* (Family: Histeridae), a small oval black beetle ca. 3 mm long. Adults and larvae feed on house fly eggs and early-instar larvae, foraging in the surface layers of manure.

The most common predaceous mite in poultry manure is *Macrocheles muscaedomesticae* (Family: Macrochelidae), a reddish-brown mite ca. 1 mm long that feeds on house fly eggs and first-instar larvae. A single mite can consume up to 20 house fly eggs per day. These mites forage externally on the manure pile, particularly at the peak in fresh manure where fly oviposition is concentrated. Large mite populations can substantially reduce house fly numbers, so mite conservation efforts can produce significant benefit.

Another predaceous mite, *Fuscuropoda vegetans* (Family: Uropodidae), forages deeper in the manure, preying on first-instar fly larvae and complementing activity of macrochelid mites at the surface.

Parasitic wasps, or parasitoids, are tiny wasps that lay their eggs in fly pupal cases, killing the developing flies. Wasp species have predilections for specific fly groups, and most filth flies have parasitoids adapted to utilize their puparia. Naturally-occurring parasitoid populations inhabit virtually all poultry facilities, but because they are extremely small (ca. 1–2 mm), parasitic wasps are seldom noticed. A female wasp seeks for a fly puparium, then inserts her ovipositor, and deposits an egg on the pupa within. The wasp egg hatches and the wasp larva consumes the fly pupa, pupates within the puparium, and eventually emerges as an adult parasitoid.

Even though these parasitoids occur naturally wherever flies are found, parasitism rates are low and fly populations are not adequately suppressed. Control programs sometimes implement mass releases of commercially reared parasitoids to augment extant populations. For successful releases, parasitoid species must be matched to the release area, as some species are adapted for xeric conditions while others are best fitted for more humid climates. When parasitoids are used, manure management can enhance their effectiveness, and insecticide use must be minimized to avoid adverse impacts on the parasitoids.

Chemical Control

Insecticides and acaricides can play an important role in poultry IPM programs, if they are used in concert with other components of the management plan. Monitoring of pest populations serves to evaluate control effectiveness, as well as alerting personnel when interventions are required. Keeping accurate records allows better future planning, with seasonal cycles and needed actions noted. Poor timing, indiscriminate pesticide use, poor manure management, inadequate moisture control, and lapsed sanitation will increase pest populations and necessitate additional insecticide applications. Most fly insecticides are toxic to predators and parasitoids, so can result in their destruction if used indiscriminately. Selective application of these chemicals, however, can target pests with minimal impact on beneficials.

Because insecticide registrations change frequently, updated recommendations can be obtained from the cooperative extension service. Always read product labels carefully before purchase, to ensure that the product will achieve your desired result. Mix and apply as directed, do not use more than the label recommends, and follow all precautions (including personal protective gear) exactly. Remember that improper practices can lead to illegal residues, even when legal materials are used, and that it is illegal to use a pesticide in any manner inconsistent with the label.

Insecticides are available in several formulations (sprays, baits, feed additives, etc.) and target different life stages (larvicides, adulticides). Understanding the advantages and limitations of each product is essential for economic and effective use.

Space sprays, typically containing synergized pyrethrins, provide a quick adult fly knockdown in a confined space. These have very little residual activity, affecting only the flies present at the time of application.

Residual sprays are used to treat building surfaces with chemicals that persist for sustained periods, so that flies landing on these treated surfaces acquire lethal doses of the toxicant and are killed. These products should be targeted to locations where adult flies are known to congregate and spend time, such as overnight resting areas and other sites marked by fly specks.

Fly baits combine a stomach poison with an attractive food (such as sugar) to lure in flies and get them to consume the material. They are excellent selective adulticides for suppressing low fly populations, especially once numbers have been reduced using a space spray. Baits should be placed where adult flies have access to them, but with minimal contamination of predator and parasitoid habitat. In high-rise houses, baits should be distributed upstairs. In addition to locating baits so that they are inaccessible to children and other nontargets, they must be used in a way to ensure they will not be eaten by birds or contaminate their feed.

Larvicides include traditional organophosphate and pyrethroid insecticides formulated for use in the manure as well as insect growth regulators (IGRs). One IGR, cyromazine, is available as both a spray and a feed-through. This compound is selective, not affecting predators and parasitoids. However, because the feed-through material ensures that all manure in the house is contaminated, and that all maggots are therefore exposed to treated manure, this formulation has resulted in development of insecticide resistance in many house fly populations. No larvicide can be expected to substitute for proper water and manure management. Larvicide sprays are formulated for direct application to the manure surface to kill maggots. Since they will destroy predators and parasitoids in the manure, larvicides should be used only as spot treatments, targeting trouble spots in the manure where maggot numbers are high. This usage pattern will minimize impact on overall biological control while slowing resistance development.

In general, reliance on chemical control of flies is decreasing due to rising application costs, increased environmental concerns and constraints, regulatory limitations, development of resistance in the target pest, and a paucity of new insecticide products.

Fly Population Monitoring in Control Programs

Control decisions should be made based on population monitoring by a standardized, quantitative method that suits the situation. Objective sampling methods include baited jug traps, sticky ribbons, and spot cards. Several sampling devices should be placed within the house, in areas of high fly activity, and checked weekly. Weekly counts should be recorded, both as a means of signaling need for control interventions and as verification of control effectiveness.

Production practices and facilities significantly determine the types of pests that can persist in animal housing. For instance, rearing laying hens in elevated wire cages removes them from exposure to parasites and pathogens in the manure, effectively breaking the disease cycle. Minimizing harborage reduces incidence of periodic ectoparasites such as bedbugs and *Dermanyssus* mites.

Rodents

Rodents are common pests in and around poultry facilities but are not unique to poultry. Rodents are capable of extensive damage to facilities, feed and poultry unless an effective control program is implemented and maintained. Rodents undermine foundations by burrowing and destroy curtains and insulation. Rodents eat and contaminate feed, increasing feed costs and adversely affecting feed conversion. These pests serve as reservoirs for a variety of diseases and ectoparasites, and may directly attack and cause injury to birds.

Rats

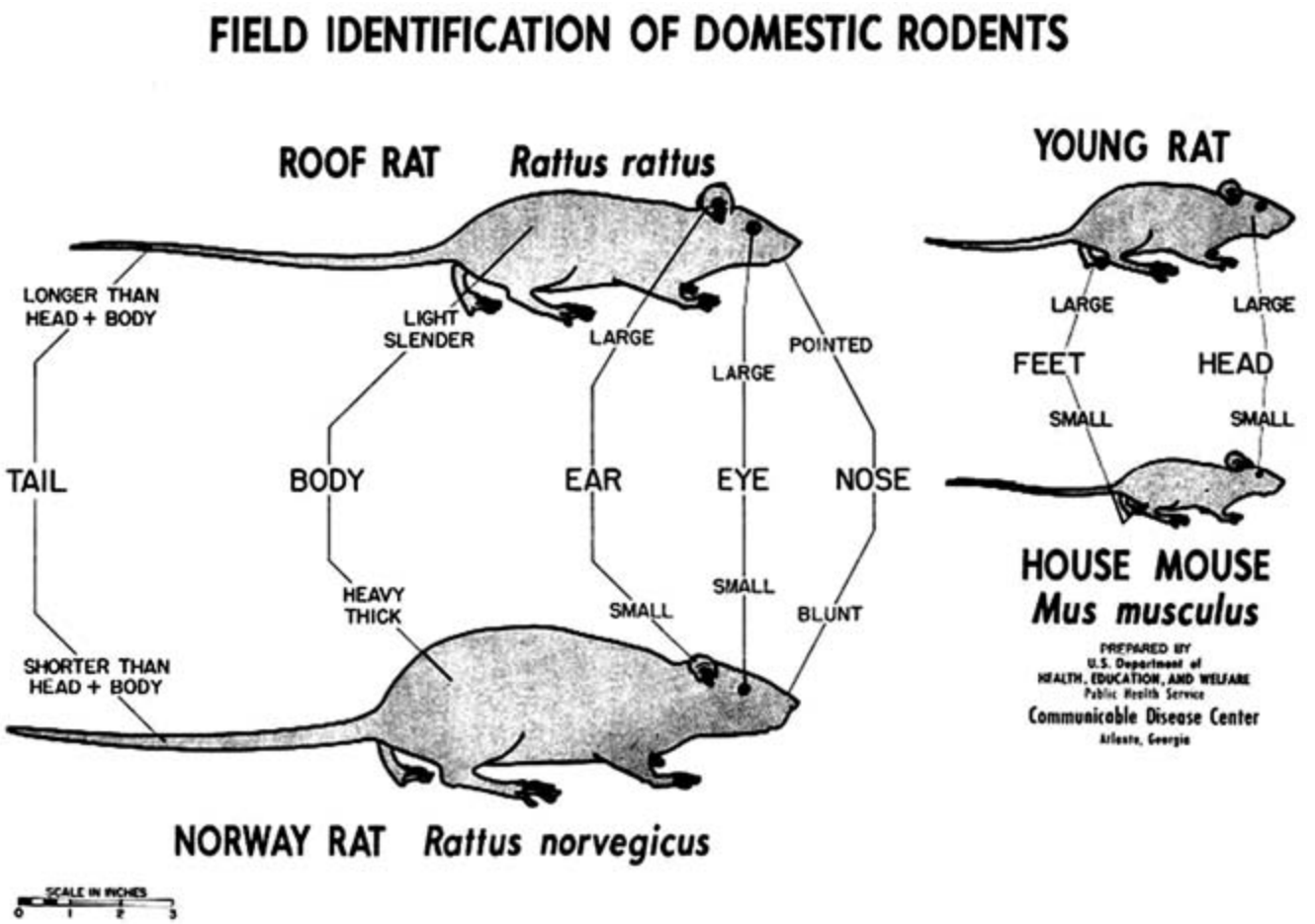
There are two main species of rats commonly found on poultry farms. These are the Norway rat (*Rattus norvegicus*) and the roof rat (*Rattus rattus*). Norway rats are easily distinguished from roof rats by their size and their nesting habits. The Norway rat is large (10–17 oz) and its tail is shorter than its body. They nest in soil outside of buildings and under concrete foundations. The roof rat is smaller (6–12 oz) and its tail is longer than its head and body combined. It nests high in buildings, lofts, ceiling insulation, etc., and in trees. Both of these rats multiply rapidly, with 3–7 litters per year. Rats breed at 3–5 months of age and the gestation period is about 3 weeks. A population of 200 adult rats reportedly consume up to 25 lbs of feed daily. Rats are largely nocturnal in feeding and other activities. Breeding is most frequent during spring and fall. Rats tend to migrate indoors in cooler weather.

Mice

The house mouse (*Mus musculus)* is the most common mouse in and around poultry facilities and wherever food, water, and harborage are available. Mice are often active throughout the day, but peak activity is at dusk or dawn. Mice burrow into the ground, insulation in the walls, rolled-up curtains, and elsewhere. Mice reproduce at an age of 6–8 weeks, and the gestation period is about 3 weeks. They produce 5–6 young per litter, and produce 5–8 litters per year. Mice tend to breed throughout the year, with no seasonal variation.

Rodent Control

Effective rodent control consists of a combination of three methods: 1) rodent-proofing of facilities; 2) sanitation and facility management; 3) trapping; and 4) effective use of rodenticides. Rodent-proofing a poultry facility is difficult, but access to buildings can be limited by patching holes in the walls and foundations, and proper screening. Sanitation simply involves cleaning up around facilities. Rodents are secretive creatures; they do not like to move about in open areas. Therefore, mowing grass and weeds on a regular basis creates a less favorable habitat. Remove piles of old wood, unused equipment, brush, discarded feed, and any other debris to make the area less attractive to ro-



26.14. Field identification of domestic rodents.

dents. Unroll house curtains twice a week even during summer months to disturb mice nesting there.

After implementing management procedures, consider an appropriate rodenticide program. Rodents can be killed with baits, fumigation, trapping, or even shooting. A properly conducted baiting program is usually the easiest and most effective means for killing rodents. Many products are marketed as rodenticides. These are of several types (Table 26.1). The largest class is the anticoagulants, which includes brodifacoum, difethialone, bromadiolone, chlorophacinone, diphacinone or warfarin. Other types include bromethalin, a metabolic inhibitor; cholecalciferol, which causes a calcium imbalance; and zinc phosphide, a metabolic poison. These products are sold under a variety of trade names. Some are effective as single doses or feedings, while others require multiple doses for effectiveness. Many of these products are available over-the-counter, but others such as zinc phosphide are highly toxic and are restricted to application by licensed pest control personnel.

For any baiting program to be effective, rodents must consume a lethal amount of the bait. Placement of baiting stations is important. Random placement of bait is rarely effective. Rodents will not seek out bait if other food is readily available. Thus, placement of bait in or close to the harborage rather than near their regular food source will be more effective. For a complete discussion of bait stations, consult pest control manuals. When using multiple-dose rodenticides, the bait should be replenished daily until the bait is no longer taken. If single-dose anticoagulants are used, the active burrows should be baited for 2 consecutive days. The burrows should be filled 4–5 days later to prevent easy recolonization. Baits are also sold in block form, which can be nailed or wired to rafters.

Acute rodenticides should be used only in cases of high rodent population when poultry and feed are no longer present. The facilities should be closed so that no animals other than rodents will have access to the bait. Bait placement is not so critical if the rodents' normal food source has been removed. All bait stations must be removed after use. Some rodenticides are available as tracking powders. These are intended to be picked up on the feet, fur, and tails of rodents, then ingested later during grooming.

An important consideration in the use of rodenticides is that resistance to some types of compounds is widespread. Even in sensitive populations, resistance may develop after repeated use of a single type of product. For long-term effectiveness of a program, it is important to rotate between types of compounds.

Table 26.1. Commonly used rodenticides (USA).

Type	Generic name	Required feeding	LD50 (mg/kg)
Anticoagulant	brodifacoum	single	house mouse, .27–.86 Norway rat, .27 roof rat, .65–.73
	difethialone	single	house mouse, .47 Norway rat, .51 roof rat, .38
	bromadiolone	single	mouse, 1.75 Norway rat, 1.125
	chlorophacinone	multiple	house mouse, 1.06 Norway rat, 2.1–20.5 roof rat, 15.0
	diphacinone	multiple	rat, 1.86–2.88
	warfarin	multiple	rodents, 3.0
Metabolic inhibitor	bromethalin	single	house mouse, 5.0 roof rat, 6.6 Norway rat, 2.0
Calcium metabolism	cholecalciferol	single	rat, 42.0
Metabolic poison	zinc phosphide	single	rat, 45.7

Since there are many commercial products containing identical chemicals, it is important to be aware of the active ingredient and to switch between unrelated products. For instance, switching from one anticoagulant to another will not improve effectiveness against anticoagulant-resistant rodents.

Fumigants used for rodent control include methyl bromide gas and chloropicrin. These are extremely hazardous to humans and nontarget animals, and should be applied only by professionals.

Claims of rodent control by ultrasonic or electromagnetic devices remain unproven in controlled studies.

Overall, the control program must be a continuous effort to be effective. Implementation of a control program after a severe problem develops will require a great deal of effort and expense. Small rodent populations are much easier to control and less expensive. Regular monitoring for rodent activity is essential. Look for rodent signs both inside and outside at least every 2 weeks and use bait as soon as activity is observed.

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Chapter 27

Internal Parasites

Introduction

Larry R. McDougald

Trematodes, acanthocephalans, cestodes, and nematodes are important parasites of poultry. In many locales the modern technology of rearing poultry has dramatically changed the importance of many species. Parasites with complicated life cycles, involving intermediate hosts such as insects or snails, were virtually eliminated when commercial poultry were brought inside. Today, only a handful of these parasites are important in commercial poultry, although many are found in small flocks reared in natural environments. A rich fauna of internal parasites can be found by examination of birds from backyard flocks, feral, or free-range birds. Wild birds of nonpoultry species have an abundant fauna of worms of all types. Many are important in the commercial production of game birds. A few nematodes, such as the ascarids, cecal worms, and capillarids, have direct life cycles and are fecund enough to prosper in the poultry house environment, particularly where management does not require frequent cleanouts. Some cestodes are important, even though they use an intermediate host, because the host also prospers in the poultry house environment.

Control of these parasites is difficult but important. Even though there have been no new products registered specifically for control of worms in chickens in recent years, excellent products approved for use in other animals can be used off-label by veterinary prescription. Even though mild infections of the worms cause little damage, some bring in other diseases, such as the well-known relationship of *Heterakis gallinarum* with *Histomonas meleagridis.* Recent widespread clinical outbreaks of blackhead disease (histomoniasis) underscore the need for advances in this area. Cestode infestations tend to build up when beetle control is lax, signaling a need for better overall management. Among anthelminthics, few have been approved for use in poultry. Recent changes in the FDA's regulation of off-label use of products by veterinarians provide some relief by allowing poultry producers to take advantage of modern products.

Nematodes and Acanthocephalans

Thomas A. Yazwinski and Christopher A. Tucker

Introduction

Nematodes constitute the most important group of helminth parasites of poultry in the number of species, the number of animals infected, and the amount of damage done. The trematodes, cestodes, and acanthocephalans are of relatively minor importance in commercial poultry, but are important in other birds.

This chapter is designed to aid the diagnostician in identifying predominant nematodes of poultry throughout the world. Those reported in chickens raised in the United States are listed in Table 27.1; those from other domestic poultry and/or commercially raised game birds from the United States are listed in Table 27.2. (Nematodes from areas other than North America are mentioned in the text but are not listed in the tables.) Avian nematodes often have a broad host range. Accordingly, nematodes found in wild birds may constitute a hazard for commercially raised birds (see Table 27.3). For a more detailed description of individual species, see the references listed in the previous editions of this book (62, 80) or other reviews (1, 12, 14, 16, 47). A checklist and descriptions are available for parasites reported from the bobwhite quail and waterfowl (43, 48). Additionally, the internal and external parasites of ratites are thoroughly discussed by Craig and Diamond in *Ratite Management, Medicine and Surgery* (73).

The genus and species names used in this chapter are those of Yamaguti (83), except where usage by recognized authorities supersedes his classification. Yamaguti described 25 families of nematodes from 9 orders in avian species; 13 of these families (Strongyloididae, Trichuridae, Syngamidae, Trichostrongylidae, Subuluridae, Heterakidae, Ascarididae, Spiruridae, Thelaziidae, Gnathostomatidae, Physalopteridea, Acuariidae, and Dipetalonematidae) contain species that infect poultry. Levine (46) used a

We thank previous authors of this chapter, Robert Norton and Michael Ruff, as well as previous authors, for their contributions to earlier versions of this work.

Table 27.1. Nematodes reported from chickens in the United States.

Nematode	Location	Intermediate Host	Other Definitive Host
Baylisascaris procyonis	Brain		Raccoons (accidental parasite in chicken, turkey, partridge, quail)
Oxyspirura mansoni	Eye	Cockroach	Turkey, duck, grouse, guinea fowl, peafowl, pigeon, quail
Syngamus trachea	Trachea	None	Turkey, goose, guinea fowl, pheasant, peafowl, quail
Capillaria contorta	Mouth, esophagus, crop	None or earthworm	Turkey, duck, guinea fowl, partridge, pheasant, quail
C. annulata	Esophagus, crop	Earthworm	Turkey, goose, grouse, guinea fowl, partridge, pheasant, quail
Gongylonema ingluvicola	Crop, esophagus, proventriculus	Beetle, cockroach	Turkey, partridge, pheasant, quail
Dispharynx nasuta	Proventriculus	Sowbug	Turkey, grouse, guinea fowl, partridge, pheasant, pigeon, quail
Tetrameres americana	Proventriculus	Grasshopper, cockroach	Turkey, duck, grouse, pigeon, quail
T. fissispina	Proventriculus	Amphipod, grasshopper, cockroach, earthworm	Turkey, duck, goose, guinea fowl, pigeon, quail
Cheilospirua hamulosa	Gizzard	Grasshopper, beetle	Turkey, grouse, guinea fowl, pheasant, quail
Ascaridia galli	Small intestine	None	Turkey, duck, goose, quail
Capillaria anatis	Small intestine, cecum, cloaca	None	Turkey, duck, goose, partridge, pheasant
C. bursata	Small intestine	Earthworm	Turkey, goose, pheasant
C. caudinflata	Small intestine	Earthworm	Turkey, duck, goose, grouse, guinea fowl, partridge, pheasant, pigeon, quail
Capillaria obsignata	Small intestine	None	Turkey, goose, guinea fowl, cecum pigeon, quail
Heterakis gallinarum	Cecum	None	Turkey, duck, goose, grouse, guinea fowl, partridge, pheasant, quail
Subulura brumpti	Cecum	Earwig, grasshopper, beetle, cockroach	Turkey, dove, duck, grouse, guinea fowl, partridge, pheasant, quail
S. strongylina	Cecum	Beetle, cockroach, grasshopper	Guinea fowl, quail
Strongyloides avium	Cecum	None	Turkey, goose, grouse, quail
Trichostrongylus tenuis	Cecum	None	Turkey, duck, goose, guinea fowl, pigeon, quail

Table 27.2. Nematodes reported from poultry or commercially raised game birds other than chickens.

Nematode	Location	Intermediate Host	Other Definitive Host
Cyathostoma bronchialis	Trachea	None or earthworm	Turkey, duck, goose, (chicken)
Cymea colini	Proventriculus	Cockroach	Turkey, grouse, prairie chicken, quail, (chicken)[a]
Tetrameres crami	Proventriculus	Amphipod	Duck
Microtetrameres helix	Proventriculus	Grasshopper	Pigeon
Amidostomum anseris	Gizzard	None	Duck, goose, pigeon
A. skrjabini	Gizzard	None	Duck, pigeon, (chicken)
Ascaridia columbae	Small intestine	None	Pigeon, dove
A. dissimilis	Small intestine	None	Turkey
A. numidae	Small intestine	None	Guinea fowl
Omithostrongylus quadriradiatus	Small intestine	None	Pigeon, dove
Heterakis dispar	Cecum	None	Duck, goose
H. isolonche	Cecum	None	Duck, grouse, pheasant, prairie chicken, quail
Capillaria columbae	Large intestine	None	Pigeon, dove

[a] Experimental

Table 27.3. Nematodes reported from wild birds in the United States that pose a potential problem for poultry or commercially raised game birds.

Nematode	Location	Intermediate Host	Definitive Host
Oxyspirura petrowi	Eye	Unknown	Grouse, quail, pheasant, prairie chicken
Splendidofilaria californiensis	Heart	Unknown	Quail
Singhfilaria hayesi	Subcutaneous	Unknown	Turkey, quail
Splendidofilaria pectoralis	Subcutaneous	Unknown	Grouse
Chandlerella chitwoodae	Connective tissues	Unknown	Grouse
Aproctella stoddardi	Body cavity	Unknown	Turkey, dove, quail
Cardiofilaria nilesi	Body cavity	Mosquito	Chicken
Echinura uncinata	Esophagus, gizzard, proventriculus, small intestine	Water flea	Duck, goose
E. parva	Proventriculus, gizzard	Unknown	Duck, goose
Tetrameres pattersoni	Proventriculus	Grasshopper, cockroach	Quail
T. ryjikovi	Proventriculus	Unknown	Duck
Cymea neeli	Proventriculus, gizzard	Unknown	Turkey
C. pileata	Proventriculus	Unknown	Quail
Physaloptera acuticauda	Proventriculus	Unknown	Chicken, pheasant
Amidostomum acutum	Gizzard	None	Duck
A. raillieti	Gizzard	None	Duck, dove
Cheilospirura spinosa	Gizzard	Grasshopper	Grouse, partridge, pheasant, quail, turkey
Cymea eurycerea	Gizzard	Unknown	Pheasant, quail, turkey
Epomidiostomum uncinatum	Gizzard	None	Chicken, duck, goose, pigeon
Streptocara crassicauda	Gizzard	Amphipod	Chicken, duck
Ascaridia bonasae	Small intestine	None	Grouse
A. compar	Small intestine	None	Grouse, partridge, pheasant, quail
Porrocaecum ensicaudatum	Small intestine	Earthworm	Chicken, duck
Capillaria phasianina	Small intestine, cecum	Unknown	Partridge, pheasant, guinea fowl
C. tridens	Small intestine	Unknown	Turkey
Aulonocephalus lindquisti	Cecum, large intestine	Unknown	Quail
A. pennula	Cecum	Unknown	Turkey
A. quaricensis	Cecum	Unknown	Quail

Note: Some of these have been reported from domestic poultry outside of the United States.

similar classification but substituted Onchocercidae for Dipetalonematidae. The classification used for families in this chapter is that given in the CIH keys in a series on the nematode parasite of vertebrates edited by Anderson and Bain (3).

General Morphology of the Nematodes Used in Identification

Nematodes, or roundworms, are usually spindle shaped with the anterior and posterior ends attenuated. The body covering, or cuticle, is often marked by transverse grooves. Longitudinal folds, or alae, may be present at the anterior (cervical alae) or posterior (caudal alae, Fig. 27.14) part of the body. The latter are found on the tail of the male worm, and in the case of certain groups, are modified to form a bursa (see Fig. 27.18B). Cuticular ornamentations occasionally found on the anterior extremities take the form of spines, cordons, or shields (see Fig. 27.6A).

The mouth opening, located at the anterior end of the body, is usually surrounded by lips bearing sensory organs (Fig. 27.5A). In some nematodes, the mouth leads directly into a cavity immediately anterior to the esophagus (Fig. 27.24A). The mouth cavity may be reduced or absent in some groups of nematodes. The esophagus may be simple (consisting of 1 undivided part) or more complex (consisting of a short anterior muscular part and a long posterior glandular part). A bulb may or may not be present at the posterior end (Fig. 27.20). The intestine follows the esophagus and leads to a short rectum connecting to the anal or cloacal opening in the posterior.

Nematodes are usually sexually distinct. Sexual dimorphism is remarkably demonstrated by some species of nematodes, such as *Tetrameres americana* (Fig. 27.7), in which the elongate male worm is much smaller than the globule-shaped female. The nematode male usually can be distinguished from the female by the presence of 2 (rarely 1) chitinous structures known as spicules, located in the posterior end of the body. The spicules (Fig. 27.20) have been considered as organs for use during copulation, keeping the vulva and vagina open and, to some extent, guiding the amoeboid sperm into the female reproductive tract. Eggs or larvae are discharged through the vulva, the position of which varies considerably between genera of nematodes.

Nematode Development

Species of nematodes in poultry have either a direct or an indirect life cycle; about one-half of the species require no invertebrate intermediate hosts, whereas the others depend on such intermediate hosts as insects, snails, and slugs. Several use paratenic hosts which facilitate parasite survival and dispersal between hosts.

Nematodes normally pass through four developmental stages and four sucessive molting events (shedding of the cuticle) before adulthood.

Eggs laid by the female nematode reach the outside in the droppings, regardless of the location of the adult worms. Some eggs are embryonated before leaving the host, but most require suitable environmental conditions outside the host for embryonation and the development of infective larvae. Most eggs hatch only when consumed by a new host, but a few hatch in the environment and release free-living larvae. Eggs become embryonated within a few days to several weeks. For nematodes with direct life cycles, the definitive host becomes infected by eating free, infective larvae or embryonated eggs containing the second-stage larvae. For those with indirect life cycles, the intermediate host ingests the embryonated eggs or free larvae. The definitive host becomes infected either by eating the infected intermediate host or by injection of infective larvae by a blood-feeding arthropod. Direct life cycles predominate among nematodes affecting commercial poultry in the United States.

Nematodes

Nematodes of the Upper Digestive Tract

Capillaria annulata *Molin 1858, Capillariidae*

Hosts

C. annulata has been reported in chicken, turkey, goose, grouse, guinea fowl, partridge, pheasant, and quail. It is found in the mucosa of the esophagus and the crop.

Morphology

C. annulata are long, slender worms, similar in appearance to *C. contorta* but easily differentiated by a cuticular swelling at the back of the head (Fig. 27.1A). The male is usually 10–26 mm long and 52–74 μm wide; the tail ends in 2 inconspicuous round lateral flaps, united dorsally by a cuticular flap; the spicule sheath is beset with fine spines (Fig. 27.1B); and the spicule is 1.12–11.63 mm long. The female is usually 25–60 mm long and 77–120 μm wide; the posterior portion of the body (posterior to vulva) is about 7 times as long as the anterior portion; the circular vulva is located opposite the termination of the esophagus; and the eggs are operculated (Fig. 27.1C) and are 55–66 × 26–28 μm.

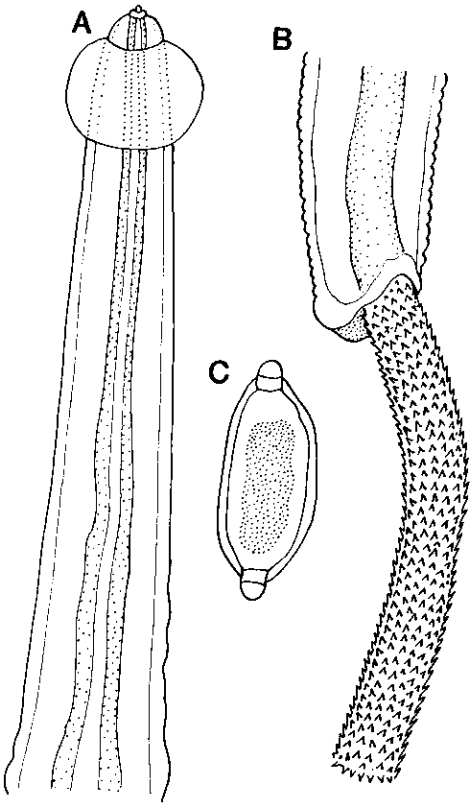
Life Cycle

Eggs pass out in the droppings of infected birds and require 24 days or more to develop. Two species of earthworms, *Eisenia foetidus* and *Allolobophora caliginosus*, serve as intermediate hosts of the crop worm (78).

Pathogenicity

The crop mucosa is thickened with enlargement of the glands, and there is inflammation of the crop and esophageal walls. In heavy infections, the inner surface of the crop becomes roughened and badly macerated. Masses of worms concentrate in the sloughing tissue.

This worm has been associated with the death of turkeys, pheasants, quail, and other gallinaceous game birds. Other signs include malnutrition, emaciation, and severe anemia.

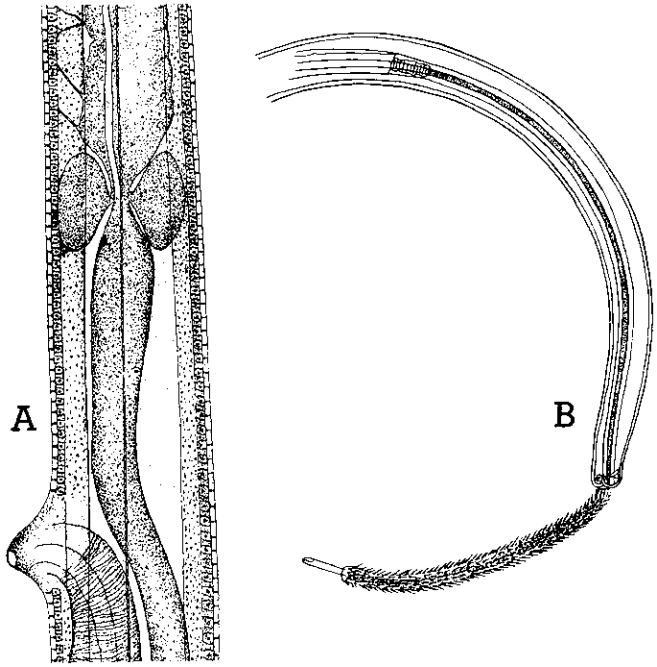


27.1. *Capillaria annulata.* A. Head end. B. Male tail. (After Ciurea) C. Egg.

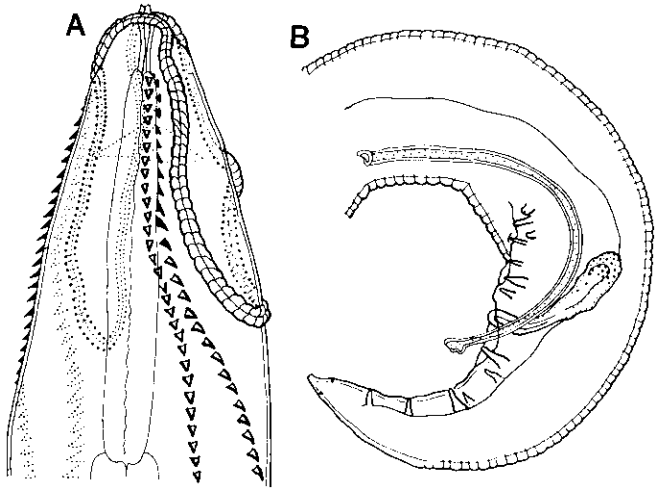
Capillaria contorta *Creplin 1839, Capillariidae*

Hosts

C. contorta has been reported in chicken, turkey, duck, guinea fowl, partridge, pheasant, pigeons and quail. It is found in the mucosa of the esophagus, the crop, and sometimes the mouth.



27.2. *Capillaria contorta*. A. Region of vulva. (After Eberth) B. Male tail. (After Travassos)



27.3. *Echinura uncinata*. A. Head. B. Male tail. (After Romanova)

Morphology

C. contorta has a threadlike body, attenuated anteriorly and posteriorly; its head is without a cuticular swelling. The male is 8–17 mm long and 60–70 μm wide; there are 2 terminal laterodorsal prominences on the tail end; the spicule is very slender and transparent, about 800 μm long; and the spicule sheath is covered with fine hairlike processes (Fig. 27.2B). The female is 15–60 mm long and 120–150 μm wide; and the vulva is prominent, circular, and situated near the beginning of the intestine (Fig. 27.2A).

Life Cycle

Eggs are deposited in tunnels in the crop mucosa and escape into the lumen of the crop and esophagus with the sloughed mucosa. They are abundant in droppings from infected birds. Approximately 1 month is required for embryonation. The life cycle is direct, and infection is initiated with the ingestion of embryonated eggs. Worms mature 1–2 months after infection.

Pathogenicity

In light infections the wall of the crop and esophagus becomes slightly thickened and inflamed. In heavy infections, a flocculent exudate covers a mucosa that is inflamed, thickened, and sloughing. The crop may become nonfunctional. In heavy infections, the worms may invade the mouth and upper esophagus.

Infected birds become droopy, weak, and emaciated. Deaths have been observed among infected wild turkeys, Hungarian partridges, and quail in the United States.

Echinura uncinata *(Rudolphi 1819) Soloviev 1912, Acuariidae*

Hosts

E. uncinata has been reported in wild and domestic ducks and geese and in wild and domestic birds in Canada.

Location

E. uncinata may be found in the mucosa of the esophagus, proventriculus, gizzard, and small intestine. There is a report of this parasite in air sacs.

Morphology

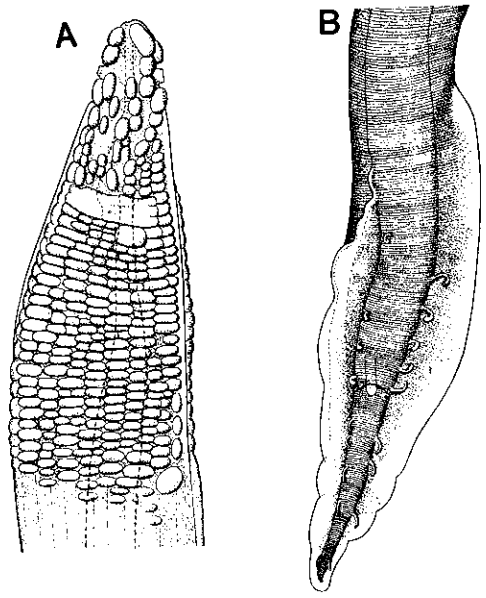
E. uncinata is similar to *Cheilospirura* and *Dispharynx*; however, the cordons are not recurrent and anastomose posteriorly (Fig. 27.3A). The male is 8–10 mm long and 300–500 μm wide; the left spicule is 700–900 μm long; and the right spicule is 350 μm long (Fig. 27.3B). The female is 12–18.5 mm long and 515 μm wide; the tail is 250 μm long; the vulva is 1.0–1.4 mm from the end of the tail; the eggs are 28–37 × 17–23 μm and embryonated when laid.

Life Cycle

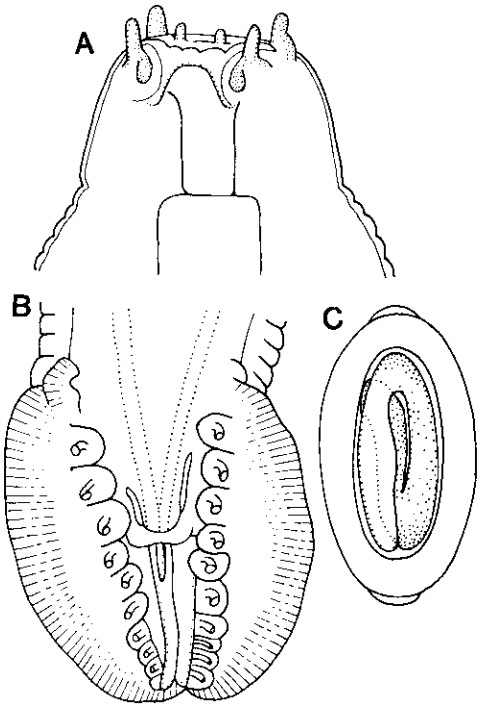
E.uncinata utilizes water fleas of the genus *Daphnia* as an intermediate host. Eggs are ingested by water fleas and release the larvae, which become infective after 12–14 days. Adults mature 51 days after ingestion of water fleas by chickens or other hosts.

Pathogenicity

Onset of mortality may be rapid, without any previous signs. Nodules may form in the proventriculus; however, in chronic infections, these may contain only inspissated pus. Birds may be emaciated and listless.



27.4. *Gongylonema ingluvicola*. A. Head. B. Male tail. (After Ransom)



27.5. *Cyrnea colini*. A. Head. B. Male tail. (After Cram) C. Egg.

Gongylonema ingluvicola *Ransom 1904, Gongylonematidae*

Hosts

G. ingluvicola has been reported in chicken, turkey, partridge, pheasant, and quail. Adults of *G. ingluvicola* may be found in the mucosa of the crop and sometimes in the esophagus and proventriculus.

Morphology

G. ingluvicola has an anterior zone of shield-like markings, with a few scattered near the head, numerous and arranged in longitudinal rows farther back (Fig. 27.4A). The male is 17–20 mm long and 224–250 μm wide; the cervical papillae are about 100 μm from the head end; the tail has 2 narrow bursal asymmetrical membranes; the genital papillae are variable in number and asymmetrical; the preanal papillae number up to 7 on the left side and up to 5 on the right side (Fig. 27.4B); the left spicule is as long or nearly as long (17–19 mm) as the body, and 7–9 μm wide with a barbed point; the right spicule is 10–12 mm long and 15–20 μm wide. The female is 32–55 mm long and 320–490 μm wide; and the vulva is 2.5–3.5 mm from the tip of the tail.

Life Cycle

The beetle *Copris minutus* and cockroaches reportedly serve as intermediate hosts for *Gongylonema ingluvicola* (15,16).

Pathogenicity

The only damage associated with these worms is local lesions in the form of burrows in the crop mucosa. The worms and their burrows appear as white convoluted tracks in the crop wall and can be confused with *Capillaria* spp. unless examined microscopically.

Cyrnea colini *Cram 1927, Habronematidae*

Hosts

C. colini has been reported in turkey, grouse, prairie chicken, and quail (and in chickens, experimentally). It is common in bobwhite quail. *C. colini* has also been reported from the turkey in Georgia and from the prairie chicken in Wisconsin and Montana. *C. colini* is found in the wall of the proventriculus, particularly at its junction with the gizzard.

Morphology

C. colini are slender yellowish white worms, similar in appearance to *Cheilospirura hamulosa* but smaller and lacking the so-called cordons or cuticular ornamentations on the anterior part of the body; the tail of the male has winglike expansions or alae (Fig. 27.5B); the head structures are complicated with four lips; dorsal and ventral lips are prominent and bear four conspicuous projecting papillae and a prominent thumblike projection (Fig. 27.5A); the lateral lips are very large, each bearing 2 digitiform processes on the inner surface and 2 winglike expansions on the lateral surface. The male is 6 mm long and 250 μm wide; the buccal cavity is 58 μm deep; the esophagus is 2 mm long; the caudal alae is nearly circular, with 10 pairs of pedunculated papillae, the anterior ones are larger than the posterior; and the spicules are very unequal; the left is 2 mm long and the right is 365–400 μm. The female is 14–18 mm long and 315 μm wide; the buccal cavity is 75 μm deep; the esophagus is about 2.8 mm long; the vulva is 915 μm anterior to the anus; and the eggs are 40.5 × 22.5 μm.

Life Cycle

The cockroach *Blattella germanica* serves as intermediate host for *C. colini* (14). Larvae develop into third stage larvae without encysting, and are fully developed by 18 days. Larvae fed to quail develop into mature worms within 41 days.

Pathogenicity

Little or no pathologic change has been observed.

Dispharynx nasuta *(Rudolphi 1819), Stiles and Hassell 1920, Acuariidae*

Hosts

D. nasuta has been reported in chicken, turkey, grouse, guinea fowl, partridge, pheasant, pigeon, quail, and a number of passerine birds in the United States.

Location

D. nasuta may be found in the wall of the proventriculus, sometimes the esophagus, and rarely the small intestine.

Morphology

D. nasuta has four wavy cuticular cordons on the anterior end, originating at the base of lips, recurrent, with the distal extremity of the cordons turning forward and extending anteriorly a short distance (Fig. 27.6A); postcervical papillae are small and bicuspid, situated between the recurrent branches of the cordons; the body is usually rolled in a spiral (Fig. 27.6B). The male is 7–8.3 mm long and 230–315 μm wide, with 5 pairs of postanal and four pairs of preanal papillae (Fig. 27.6C); the long spicule is 400 μm long, slender and curved; the short spicule is 150 μm long and navicular. The female is 9–10.2 mm long and 360–565 μm wide; the vulva is in the posterior portion of body; and the eggs are embryonated when laid.

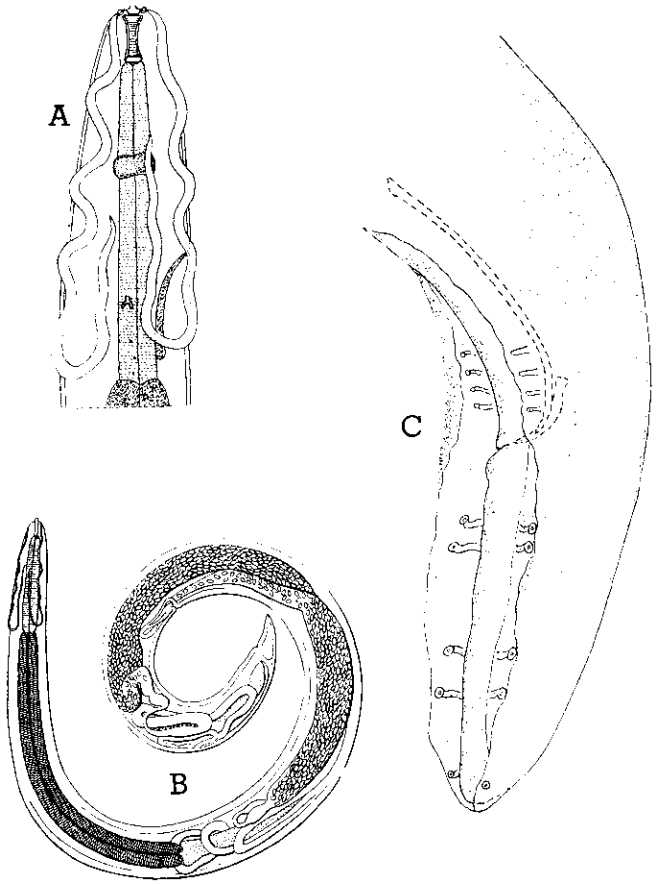
Life Cycle

The pillbug (*Armadillidium vulgare*) and the sowbug (*Porcellio scaber*) serve as intermediate hosts in experimental infections (14). Within 4 days after ingestion of embryonated eggs, larvae escape from the eggs and are found among the tissues of the body cavity. The larva completes its development to the third or infective stage in the isopod within approximately 26 days. Female worms become sexually mature and deposit eggs 27 days after ingestion by a susceptible vertebrate host.

Pathogenicity

Ulcers are often observed in the proventriculus where the worms bury their heads deep in the mucosa. In heavy infections, the wall of the proventriculus becomes tremendously thickened and macerated, tissue layers are indistinguishable, and the parasites become almost completely concealed beneath the proliferating tissue.

D. nasuta is considered the chief cause of "grouse disease" in the northeastern United States. Heavy infections have resulted in the death of carrier pigeons. Wild pigeons trapped at the Balboa Zoological Park in San Diego, California, were heavily infected with this parasite.



27.6. *Dispharynx nasuta*. A. Head. (After Seurat) B. Female (After Piana) C. Male tail. (After Cram)

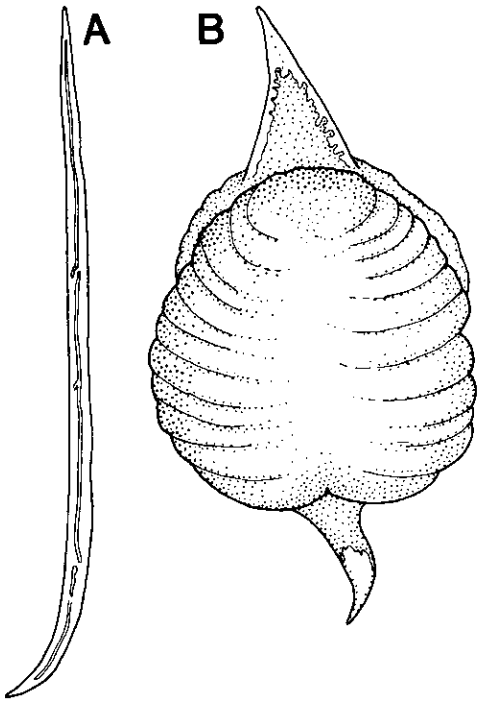
Tetrameres americana *Cram 1926, Tetrameridae*

Hosts

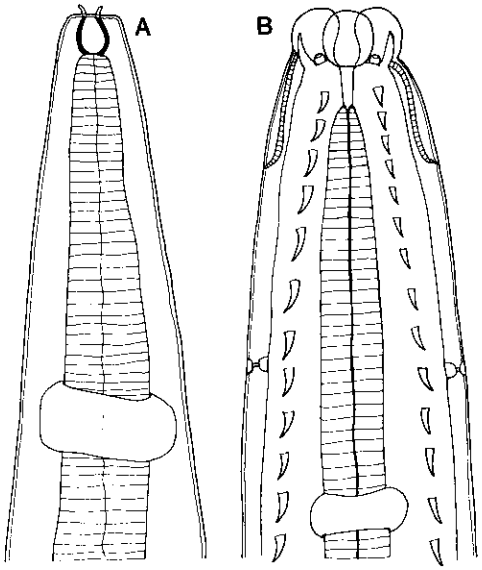
T. americana parasitizes the proventriculus of chickens, turkeys, ducks, grouse, pigeons, and quail. At necropsy, these bright red worms are often observed through the wall of the unopened proventriculus.

Morphology

There is marked sexual dimorphism (Fig. 27.7). The female is globular (Fig. 27.7B), blood red in color, with four longitudinal furrows; the uteri and ovaries are very long, their numerous coils filling the body cavity; and eggs are 42–50 × 24 μm and embryonated when laid. The female is 3.5–4.5 mm long and 3 mm wide. The mouth is surrounded with three small lips, and a buccal cavity is present (Fig. 27.8A). The male is 5–5.5 mm long and 116–133 μm wide; 2 double rows of posteriorly directed spines extend throughout the whole body length in the submedian lines; cervical papillae are present; the tail is long and slender; and there are 2 unequal spicules, 100 μm and 290–312 μm long.



27.7. *Tetrameres americana*. A. Male. B. Female. (After Cram)



27.8. A. *Tetrameres americana*, head. (Courtesy Graybill) B. *Tetrameres fissispina*, head. (Travassos)

Life Cycle

T. americana requires an intermediate host for its complete development (14). Embryonated eggs consumed by grasshoppers (*Melanoplus femurrubrum* and *M. differentialis*) or cockroaches (*Blatella germanica*) produce infective (third stage) larvae in the body cavities about 42 days later. After ingestion of the insect by birds, the larvae escape and spend at least 14 days in the gastric mucosa, molting to the fourth stage. Females contain embryonated eggs by day 45.

Pathogenicity

Infected chickens become emaciated and anemic in heavy infections. *T. americana* did not produce any damage in quail (16). In chickens, the wall of the proventriculus may be thickened, almost blocking the lumen.

T. americana has been reported both from wild (26) and laboratory-raised pigeons (31). Mild infections produced little evidence of clinical disease; heavy infections result in diarrhea, emaciation, and death.

Tetrameres crami *Swales 1933, Tetrameridae*

Host

T. crami is found in the proventriculus of wild and domestic ducks.

Morphology

T. crami is smaller than *T. americana.* The male is 2.9–4.1 mm long and 70–92 μm wide. It has a narrow curved right spicule, 136–185 μm long, and a twisted left spicule 272–350 μm long. The female is 1.5–3.3 mm long and 1.2–2.2 mm wide; its tail is 113–156 μm long; the vulva is 319–350 μm from the posterior end; and the eggs are 41–57 × 26–34 μm and embryonated when laid.

Life Cycle

The intermediate hosts of *T. crami* are the amphipods, *Gammarus fasciatus* and *Hyalella knickerbocki* (69). The larvae become infective in 29 days. Adults mature 33 days after infection.

Tetrameres fissispina *(Diesing 1861) Travassos 1915, Tetrameridae*

Hosts

T. fissispina has been reported in the proventriculus of chickens, turkeys, ducks, guinea fowl, geese, pigeons, and quail. It is more common in wild or domestic ducks, geese and wild birds and is rare in other poultry.

Morphology

T. fissispina is similar in appearance to *T. americana.* The male is 3–6 mm long and 90–200 μm wide; it has four longitudinal rows of spines along the median and lateral lines (Fig. 27.8B); and spicules are 280–490 μm and 82–150 μm long. The female is 1.7–6.0 mm long and 1.3–5.0 mm wide; its tail is 71 μm long; the vulva is 310 μm from the posterior end; and its eggs are 48–56 × 26–30 μm and embryonated when laid.

Life Cycle

Intermediate hosts include amphipods, grasshoppers, earthworms, and cockroaches. Larvae are infective by day 10. Worms mature about 18 days after ingestion. Fish may serve as a transport host.

Pathogenicity
Considerable tissue reaction occurs together with degeneration of the glandular tissue, edema, and extensive leukocyte infiltration (72).

Tetrameres pattersoni *Cram 1933, Tetrameridae*

Host
T. pattersoni is found in the proventriculus of quail.

Morphology
Bright red female *T. pattersoni* worms are found embedded in the glands of the proventriculus; males are on the surface of mucosa. The male is 4.2–4.6 mm long and 140–170 μm wide; it has a body with 2 rows of spines ending just anterior to the cloacal aperture; three pairs of lateral and four pairs of subventral spines are posterior to the cloacal aperture; and only 1 spicule exists, 1.2–1.5 mm long with conspicuous cross-striations. The female is 5 mm long and 2–2.3 mm wide; the vulva is about 235 μm from the tail end; the anus is 156 μm from tail end; there is a bulbous enlargement between the vulva and the anus; and the eggs are 42–46 × 25–30 μm.

Life Cycle
Intermediate hosts include grasshoppers (*Melanoplus femurrubrum* or *Chortophaga viridifasciata*) or cockroaches (*Blattella germanica*) in which it develops into infective third stage larvae within 24 days (15). Larvae encyst in the muscles and the mesenteries of the body cavity, each cyst containing 1–3 larvae. The tail end of the larva has a small protuberance, differing from *T. fissispina* and *T. americana.*

Pathogenicity
Worms can be so numerous that little uninfected stomach wall remains. Severe infections may cause death.

Libyostrongylus douglassii

Hosts
L douglassii is commonly found in the ostrich. It is found in the duct system of glands lining the wall of the proventriculus.

Morphology
These nematodes, as adults, are 4–6 mm in length; spicules are 140 to 160 μm long; and the strongylorin type eggs are 72 × 41 μm when laid. An additional, commonly occurring, related species is *L. dentatus*, which is slightly larger than *L. douglassii* (7–13 mm long), but which lays a similar egg.

Life Cycle
It is direct, with infection commencing upon ingestion of infective, third-stage larvae. Prepatency is approximately 30 days. Infective larvae are resistant to harsh environmental conditions on pasture for as long as 30 months.

Pathogenicity
L. douglassii is highly pathogenic in young ostriches, with reports of up to 50% mortality. The hematophagic nematodes block the ducts of proventicular glands, induce excessive mucus secretions to the point of impaction, and eventually cause an acute diphtheritic condition of the proventriculus ("vrotmaag"; South African for "rotten stomach").

Vaznema zschokkei

Host
It is found in the submucosa of the proventriculus of the rhea.

Morphology
These spirurid nematodes are 16 to 25 mm in length, with spicules approximately 10 mm in length.

Amidostomum anseris *Zeder 1800, Amidostomatidae*

Hosts
A. anseris has been reported in ducks, geese, and pigeons. In the United States, parasites have been reported from domestic geese in New York, Delaware, Pennsylvania, and Washington.

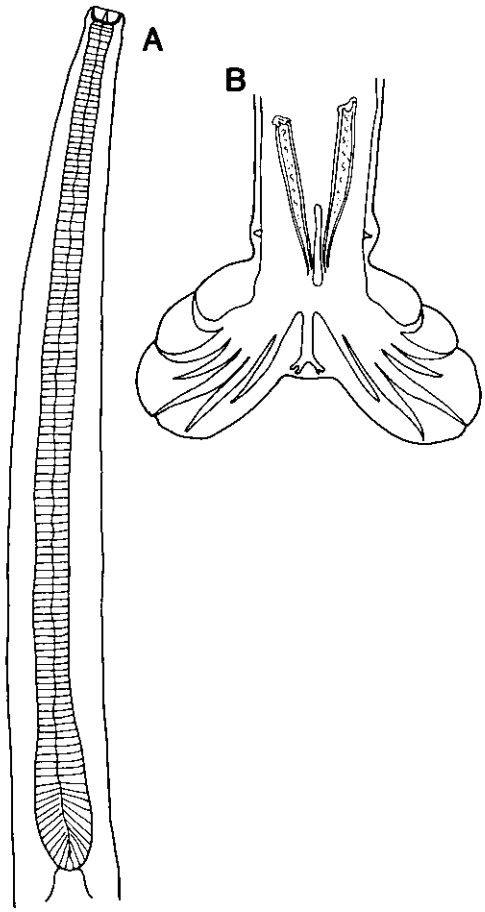
Location
A. anseris is found under the horny lining of the gizzard and less frequently in the proventriculus.

Morphology
A. anseris is slender and reddish; its short wide buccal capsule has three pointed teeth at its base (Fig. 27.9A). The male is 10–17 mm long and 250–350 μm wide; it has a bursa with 2 large lateral lobes and a small median lobe (Fig. 27.9B); its dorsal ray is short, bifurcating posteriorly with the bifurcations forked and terminating in 2 tips; the spicules are 200 μm long, slender, and cleft near their middles; the gubernaculum is slender and 95 μm long. The female is 12–24 mm long and 200–400 μm wide at the vulva, thinning toward both extremities; the vulva is in the posterior part of the body; and eggs are thin shelled, 85–110 × 50–82 μm.

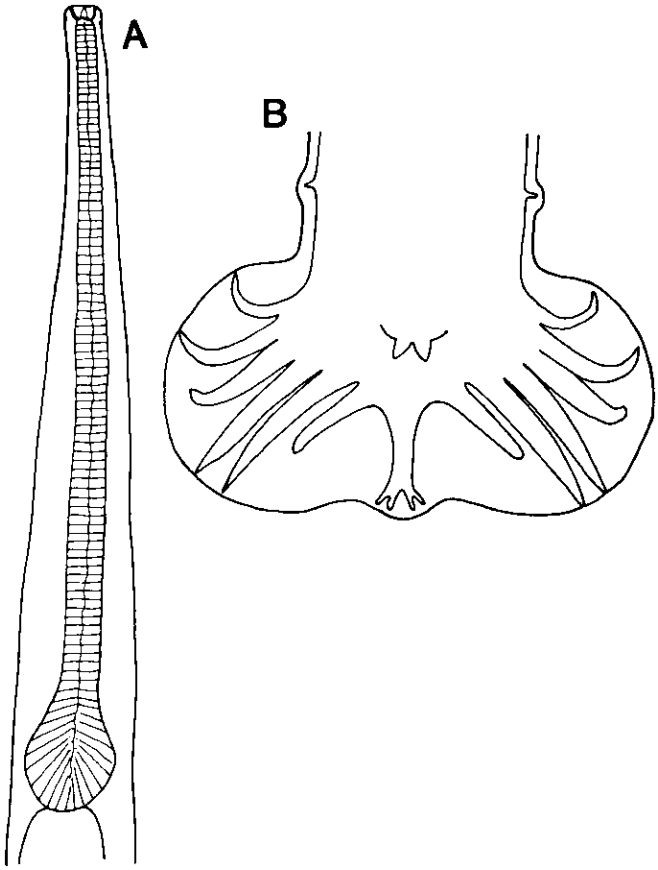
Life Cycle
Direct. Eggs are passed in a partly developed stage; active embryos develop within a few hours and hatch within a few days. Susceptible birds become infected by swallowing food or water contaminated with infective larvae, and adults worms are found within 40 days. This parasite is quite host specific; attempts to infect a variety of other hosts were unsuccessful (20).

Third-stage larvae may also infect through the skin of birds. Larvae migrate via the lungs in percutaneous infections but not in oral infections (19).

Pathogenicity
Heavy losses among geese have been attributed to this nematode. Young birds show loss of appetite, dullness, and emaciation. The lining of the gizzard of a heavily parasitized bird appears necrotic, loosened, and often detached in places. Infection may result in blood loss.



27.9. *Amidostomum anseris*. A. Anterior. (After Boulenger) B. Male bursa. (After Railliet)



27.10. A. Head, *Amidostomum raillieti.* B. Male bursa, *Amidostomum skrjabini*. (After Boulenger)

Amidostomum skrjabini *Boulenger 1926, Amidostomatidae*

Hosts

A. skrjabini is found under the horny lining of the gizzard in ducks and pigeons (and chicken, experimentally).

Morphology

A. skrjabini is smaller than *A. anseris* and can be distinguished from *A. raillieti*. The male is 7.5–8.8 mm long and 100–130 μm wide, with the bursa resembling that of *A. raillieti*; each branch of the dorsal ray is further divided into 2 equal branches (Fig. 27.10B); and spicules are 115–125 μm long. The female is 9–11 mm long and 101–120 μm wide; the vulva is 1.7–2.1 mm from the posterior end; and the eggs are 70–80 × 40–50 μm, and at the morula stage when laid (Fig. 27.10).

Life Cycle

The life cycle of *A. skrjabini* is similar to that of *A. anseris*.

Pathogenicity

A. skrjabini has been involved in clinical disease outbreaks in young ducks.

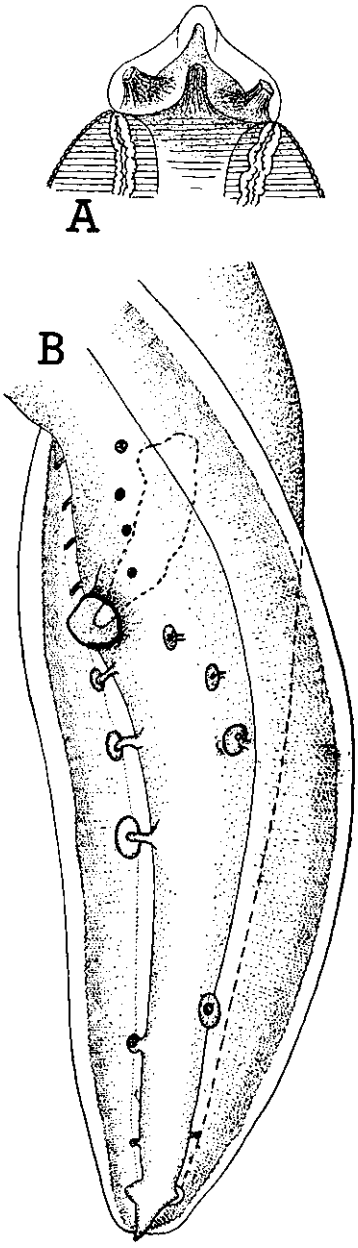
Cheilospirura hamulosa *(Diesing 1851) Diesing 1861, Acuariidae*

Hosts

C. hamulosa has been reported in chickens, turkeys, grouse, guinea fowl, pheasants, and quail. It is found under the horny lining of the gizzard, usually in the cardiac and/or pyloric regions, where the lining is soft and pliable.

Morphology

C. hamulosa has 2 large triangular lateral lips. The 4 cuticular cordons are irregularly wavy (Fig. 27.11A), and extend at least two-thirds the length of the body and sometimes almost to the posterior extremity; they do not anastomose or recur anteriorly and are characteristic of this species. The male is 9–19 mm long; spicules are unequal and dissimilar, with the left being long and slender, 1.6–1.8 mm, and the right short and curved, 180–200 μm. The tail is tightly coiled; 2 very wide caudal alae are present; and 10 pairs of caudal papillae exist (Fig. 27.11B). The female is 16–25 mm long; the vulva is slightly posterior to the middle of body; the tail is pointed; and eggs are 40 × 27 μm and embryonated when laid.

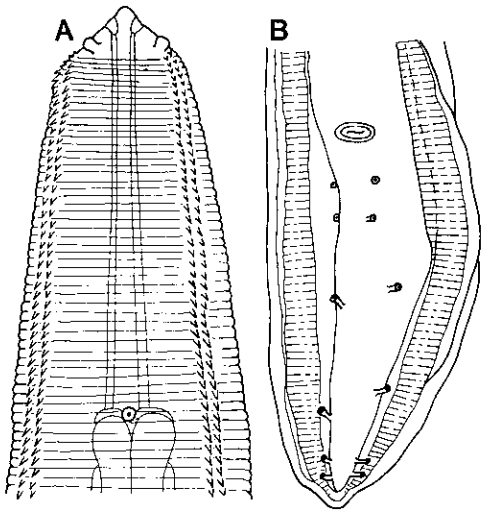


27.11. *Cheilospirura hamulosa*. A. Head. (After Drasche) B. Male tail. (After Cram)

Life Cycle

Grasshoppers, beetles, weevils, and sandhoppers serve as intermediate hosts (14). The infective third-stage larvae develop in the arthropod's muscles. Larvae are recognized by the 2 prominent liplike structures at the anterior end of the body, the dorsal curvature of the posterior portion of the body, and the 4 digitiform processes at the tip of the tail.

Larvae are infective for chickens as early as 22 days after entering the intermediate host, and reach maturity in the bird in about 76 days.



27.12. *Cheilospirura spinosa*. A. Head. B. Male tail. (After Cram)

Pathogenicity

Presence of small numbers of these worms causes little damage, although the lining of the gizzard may show small local lesions that also involve the muscular tissue. Soft nodules enclosing parasites may be found in the muscular portion of the gizzard. In heavy infections, the wall of the gizzard may be seriously damaged.

Cheilospirura spinosa *Cram 1927, Acuariidae*

Hosts

C. spinosa has been reported in grouse, partridge, pheasant, quail, and wild turkey. It is found in the gizzard underneath the corneous lining.

Morphology

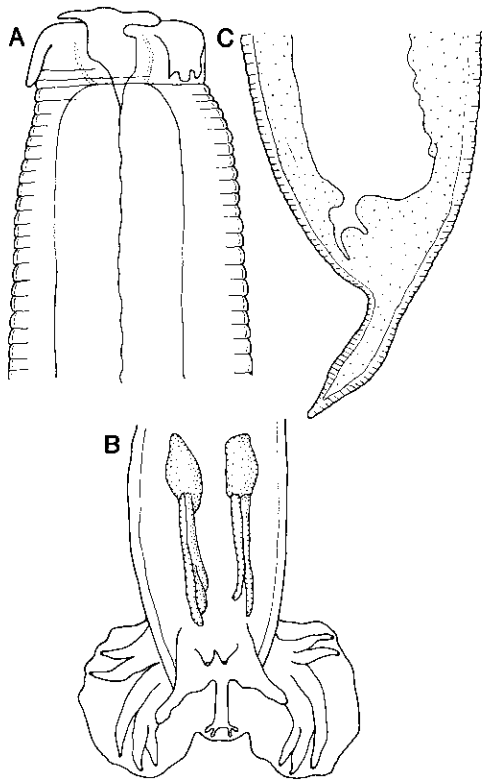
C. spinosa has four spiny cordons originating in pairs between the lips (Fig. 27.12A), not extending beyond the anterior third of the esophagus. The male is 14–20 mm long and 183–232 μm wide; spicules are unequal and very dissimilar; one being 660–720 μm long, and the other being 192 μm long. The caudal alae are broad and similar in appearance to *C. hamulosa* (Fig. 27.12B). The female is 34–40 mm long and 315–348 μm wide; the vulva is anterior to the middle of body; the anus is 250–300 μm from the posterior end; and eggs are 39–42 × 25–27 μm.

Life Cycle

Grasshoppers serve as the intermediate host, where development is similar to that of *C. hamulosa.* The tail structures on the third-stage larvae are similar in *C. spinosa*, rather than dissimilar as is the case with *C. hamulosa.* In the bobwhite quail, fourth stage larvae are found underneath the gizzard lining 14 days after infection (14). Worms with fully developed sexual characteristics are seen by 32 days.

Pathogenicity

Mild infections produce few problems in quail, although tortuous paths are found between the lining and muscle tissues of the giz-



27.13. *Epomidiostomum uncinatum*. A. Head. B. Male tail. C. Female tail. (After Skrjabin)

zard. In heavy infections, the gizzard lining may become hemorrhagic and necrotic, with marked proliferative changes in the gizzard wall.

Epomidiostomum uncinatum *(Lundahl 1841) Seurat 1918, Amidostomatidae*

Hosts

E. uncinatum has been reported in ducks, geese, and pigeons (and chickens, experimentally). It is found under the horny lining of gizzard.

Morphology

E. uncinatum differs from *Amidostomum* in that the buccal capsule contains no teeth, and the head has a pair of nodules (Fig. 27.13A). The male is 6.5–7.3 mm long and 150 μm wide; spicules are 120–130 μm long (Fig. 27.13B), dividing to form three terminations. The female is 10–11.5 mm long and 230–240 μm wide; the tail is 140–170 μm long (Fig. 27.13C); the vulva is 2.2–3.2 mm from the posterior end; and eggs are 74–90 × 45–50 μm.

Life Cycle

There is no intermediate host. Third-stage larvae are infective 4 days after hatching (44).

Sicarius uncinipenis *and* S. waltoni

Host

Rhea, gizzard.

Morphology

These spiruid nematodes resemble *Habronema* spp. as seen in the horse. *S. uncinipenis* is 18–30 mm in length. Males have unequal spicules; approximately 3 and 0.7 mm in length. *S. waltoni* is slightly smaller, < 25 mm in length and with unequal spicules 2.5 or 0.35 mm in length.

Nematodes Found Primarily in the Small Intestine

Ascaridia bonasae *Wehr 1940, Ascaridiidae*

Host

Grouse, primarily the small intestine.

Morphology

Several authors have apparently confused *A. bonasae* with *A. galli,* although *A. bonasae* is small and does not infect the chicken. The male is 10–35 mm long, and spicules are 1.8–2.7 mm long and equal. The female is 30–50 mm long.

Life Cycle

The direct life cycle of *A. bonasae* is similar to that of *A. galli*.

Ascaridia columbae *(Gmelin 1790) Travassos 1913, Ascaridiidae*

Hosts

Pigeons and doves. Usually found in the lumen of the small intestine, but sometimes in the esophagus, proventriculus, gizzard, liver, or body cavity.

Morphology

The *A. columbae* male is 50–70 mm long; spicules are 1.2–1.9 mm long and equal. *A. columbae* has a fourth pair of ventral papillae located adjacent to the anus (Fig. 27.14A). The female is 20–95 mm long.

Life Cycle

Direct, similar to that of *A. galli*. Second-stage larvae frequently penetrate the intestinal mucosa and reach the liver and lungs but do not develop further (81). Worms mature in about 37 days in the intestine.

Pathogenicity

Granulomatous lesions with leucocyte infiltration may result from invasion of the liver by larvae. Otherwise, pathogenicity is low.

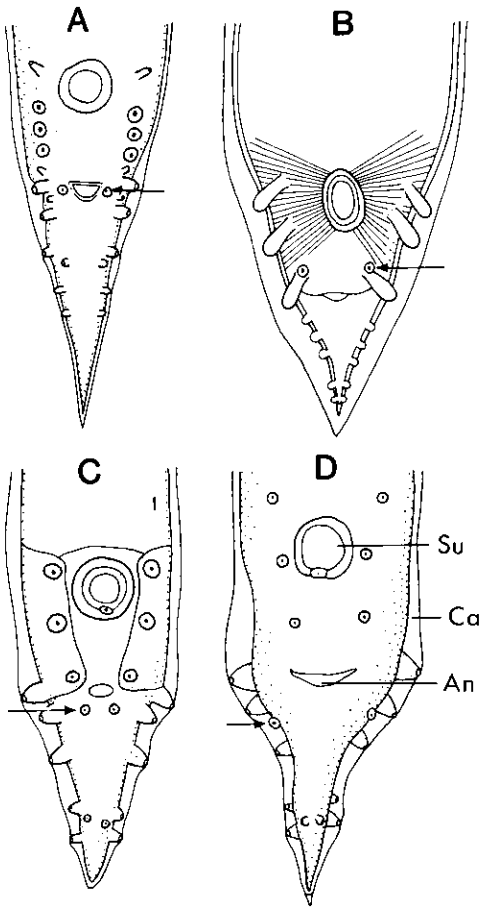
Ascaridia compar *Schrank 1790, Ascaridiidae*

Hosts

Grouse, partridges, pheasants, and quail.

Morphology

The male is 36–48 mm long; spicules are 1.8 mm long; and there are four pairs of preanal papillae, two near the preanal sucker, two just anterior to the anus (Fig. 27.14B). The female is 84–96 mm long.



27.14. Male tails. A. *Ascaridia columbae*. (After Wehr and Hwang). B. *Ascaridia compar*. (After Linstow) C. *Ascaridia dissimilis*. D. *Ascaridia galli*. (After Wehr)

Life Cycle

The life cycle of *A. compar* is similar to that of *A. galli.*

Ascaridia dissimilis *Perez Vigueras 1931, Ascaridiidae*

Host

A. dissimilis is commonly reported in turkeys and is the only nematode parasite of confinement-raised, commercial turkeys in the USA that exists in patent infections.

Location

The lumen and wall of the small intestine.

Morphology

This worm resembles *A. galli*. Only males can be identified accurately based on caudal papillae and spicule tips. The male is 35–65 mm long; spicules are 1.3–2.2 mm long, and the distal ends of the spicules are rounded; the first pair of preanal papillae are opposite the preanal sucker; the ventral pair of postanal papillae are only slightly separated and just behind the anus (Fig. 27.14C). The female is 50–105 mm long.

Life Cycle

The direct life cycle of *A. dissimilis* is similar to that of *A. galli.* Eggs embryonate in 14–30 days given adequate moisture and temperature. After ingestion, the second stage larvae hatch and are free in the mucous layer of the small intestine for a few days before molting into the third stage. The larvae then either remain in the mucus or invade the mucosa and submucosa for an invariable period of time. Very high levels of third-stage larvae are common in commercial turkeys, as this stage is capable of developmental arrest—providing a reservoir population of this nematode in the small intestine. Eventually, the developing third-stage larvae will accumulate in the mucous layer where they molt to the fourth larval stage. An extensive loss of nematode numbers occurs naturally between the third and fourth stages. Final molt to the adult stage occurs 21 days or more after infection. Natural infections of turkeys are characterized by large numbers of third-stage larvae (upward of 2000 per bird) and low numbers of all other stages.

Pathogenicity

Mortality and lowered productivity are associated with *A. dissimilis* in turkeys (34, 53). Surveys of commercial flocks in the south-central United States indicated that a high percentage of turkey flocks are heavily parasitized with *A. dissimilis* (54). Aberrant migration of *A. dissimilis* larvae may cause hepatic foci and granulomas as the worms migrate through the portal circulatory system into the liver (55). Anorexia; intestinal inflammation; and loss of metabolites, fluids, and proteins lead to lowered feed efficiency and poor performance. In addition, given the extent of mucosal invasion and tissue inflammation, infection of turkeys with *A. dissimilis* also coincides with depression of immune competence and facilitates access of opportunist pathogens into the gut tissue and beyond.

Ascaridia galli *Schrank 1788, Ascaridiidae*

Synonyms

A. lineata Schneider 1866; *H. granulosa* Linstow 1906.

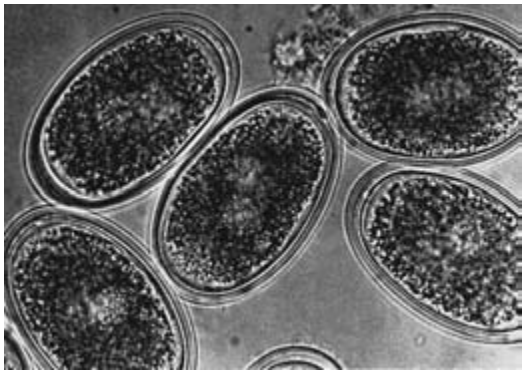
Hosts

A. galli has been reported in chickens, turkeys, doves, ducks, and geese and is normally found in the small intestine.

With aberrant migration, it is occasionally found in the bird's esophagus, crop, gizzard, body cavity, oviduct, and egg.

Morphology

A. galli are large, thick, yellowish white worms; their head has three large lips. The male is 50–76 mm long and 1.21 mm wide; the preanal sucker is oval or circular, with a strong chitinous wall with a papilliform interruption on its posterior rim; the tail has narrow caudal alae or membranes and 10 pairs of papillae; the first pair of ventral caudal papillae is anterior to the preanal sucker; the fourth pair is widely separated (Fig. 27.14D, compare with *A. dissimilis*); and spicules are nearly equal and narrow, with blunt ends and slight indentations. The larger female is 60–116 mm long and 1.8 mm wide; the vulva is in the anterior part of body; and the eggs are elliptical, thick shelled, and not embryonated at time of deposition (Fig. 27.15).



27.15. *Ascaridia galli* eggs freshly voided from a chicken. ×400 (Benbrook)

Life Cycle

A. galli has a simple and direct life cycle. Infective eggs hatch in the proventriculus or the duodenum of the susceptible host. The second stage larvae, after hatching, are free in the mucous layer of the duodenum for the first several days after infection. Then, a portion of their numbers penetrates the mucosa and molt to the third-larval stage. Third-stage larvae return to the lumen by approximately day 17, where they molt to the fourth stage and mature by 28–30 days of age. The life cycle of *A. galli, A dissimilis*, and other worms of this genus are very similar. In commercial birds, however, infection dynamics are quite dissimilar. Populations of the ascarids in turkeys are much greater than those seen in chickens. Additionally, infections in turkeys are predominately third larval stages and maintained at high levels for the life of the turkey. In chickens, adult stages appear to predominate with no reservoir population of developmentally arrested third stages, and a definite age resistance is manifest in the chicken with bird age inversely proportionate to infection size.

A. galli eggs may be ingested by grasshoppers or earthworms (potential, paratenic hosts) and remain infective to chickens, with no development of the larvae occurring in the invertebrate.

Under optimum conditions of temperature and moisture, eggs in the droppings become infective in 7–28 days. Eggs are resistant to low (nonfreezing) temperatures. Embryonated eggs of this worm have survived outdoors at Beltsville, MD, for up to 66 weeks (27). However, eggs are killed by a 12-hour exposure to 43°C.

Pathogenicity

A. galli infection causes weight depression in the host, proportionate to increasing worm burden (59). The nutritional state of the host is also important, because weight depression is greater with high dietary levels of protein (15%) than with low levels (12.5%) (39). In severe infections, intestinal blockage can occur. Chickens infected with a large number of ascarids suffer from loss of blood, reduced blood sugar content, increased urates, shrunken thymus glands, retarded growth, and increased mortality. However, no effects of infection on blood protein level, packed cell volume, or hemoglobin levels were found (38). *A. galli* can also synergize the effects of other disease conditions, such as coccidiosis and infectious bronchitis. *A. galli* reportedly contain and transmit avian reoviruses.

Chickens 3 months of age or older manifest considerable resistance to infection with *A. galli,* regardless of previous infection status. In older fowl, larvae may undergo little or no development after emerging from the egg (71). Larval development is arrested in the third stage at high dose rates as a result of age resistance (47). Heavier breeds, such as Rhode Island reds and white and barred Plymouth rocks, are more resistant to ascarid infections than are the lighter white leghorns and white minorcas.

The nutritional state of the bird also influences the development of immunity. Diets high in vitamins A and B (complex) increase the fowl's resistance to *A. galli.* Increasing levels of dietary calcium and lysine decreased the length and number of worms recovered (17).

One of the most striking results of infection by this nematode is the occasional finding of this parasite in the hen's egg. Numerous reports of this phenomenon have been made in the literature (60). These aberrant worms have evidently migrated from the intestine, across the cloaca, and up the oviduct, with subsequent inclusion in the egg. It is thought that this phenomomen is a result of the incomplete action of narcotizing wormers such as piperazine. Infected eggs can be detected by candling.

Ascaridia numidae *Leiper 1908, Ascaridiidae*

Host

A. numidae is a parasite of guinea fowl. It is found in the lumen of the small intestine, or sometimes the cecum.

Morphology

A. numidae are much smaller than *A. galli*. The male is 19–35 mm long; it has 10 pairs of caudal papillae, 2 of them preanal and 2 adanal; and spicules are equal, 3 mm long. The female is 30–50 mm long.

Life Cycle

Direct, similar to other ascarids. The larvae remain in the lumen for 4–14 days before penetrating the intestinal mucosa.

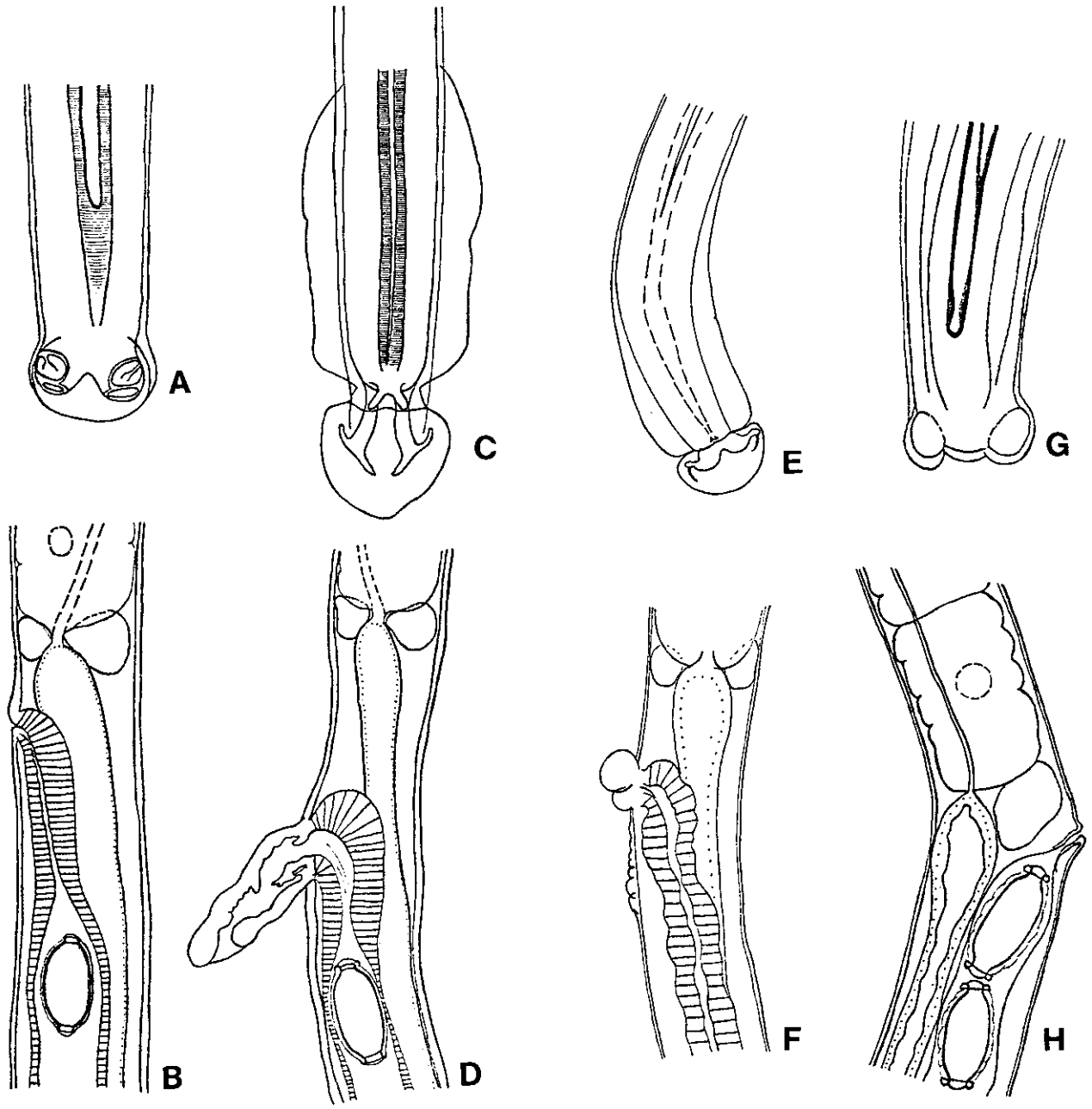
Intestinal Capillaria

Species of *Capillaria* from birds have been described under a variety of names, leading to confusion in the literature. In this chapter, names accepted by Levine (46) are used with the exception of *C. dujardini,* which is considered a synonym for *C. obsignata.* The species *C. columbae* (Rudolphi,1819) is retained for the *Capillaria* in the large intestine of pigeons that possesses a vulva with a projecting appendage (Table 27.4 and Fig. 27.16).

Capillaria obsignata *Madsen 1945, Capillariidae*

Hosts

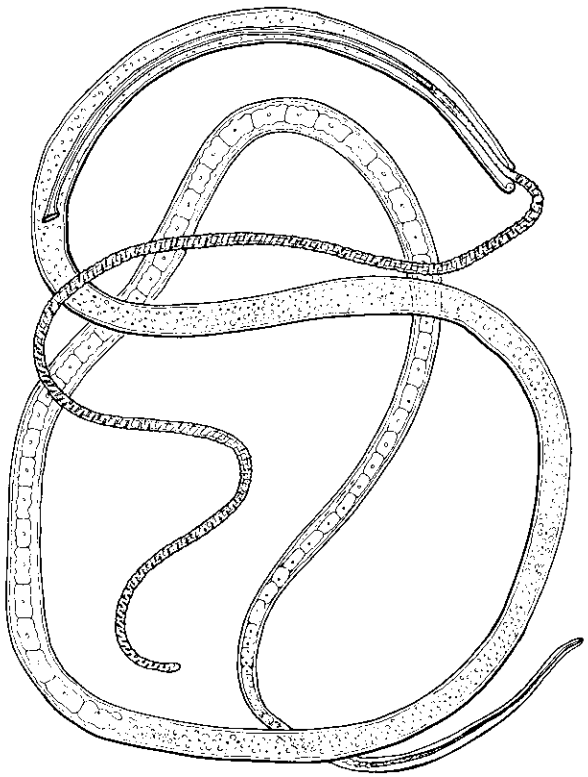
Chickens, turkeys, geese, guinea fowl, pigeons, and quail. *C. obsignata* is common in older, layer or breeder chickens. Infection levels as high as 2000 worms per bird are common with replacement pullets, levels of infection that persist through production (litter confinement). *C. obsignata* is found in the small intestine.



27.16. Male bursa (A, C, E, G) and female vulva (B, D, F, H) of *Capillaria obsignata* (A, B), *Capillaria caudinflata* (C, D), *Capillaria bursata* (E, F), and *Capillaria anatis* (G, H). (After Wakelin)

Table 27.4. Characteristics of *Capillaria* from chickens in the United States.

Characteristic	*C. anatis*	*C. bursata*	*C. caudinflata*	*C. obsignata*
Male				
Lateral caudalae	–	+	+	–
Spicule sheath	Spines	No spines	Minute spines	No spine
Female				
Vulvar appendage	None	Semicircular	Pronounced	None



27.17. *Capillaria obsignata*. (After Gagarin)

Morphology

C. obsignata is hairlike and usually spiraled in appearance (Fig. 27.17). The male is 7–13 mm long with the cloacal aperture almost terminal, and a small bursal lobe on either side, the 2 lobes connected dorsally by a delicate bursal membrane (Fig. 27.16A). The spicule is 1.1–1.5 mm long and the sheath has transverse folds without spines. The female is 10–18 mm long; the vulva is slightly prominent and slightly posterior to the union of esophagus and intestine (Fig. 27.16B). The operculate eggs are 44–46 × 22–29 μm with a reticulate pattern on the shells.

Life Cycle

C. obsignata has direct development (76). Embryonation of ova is complete in 13 days at 20°C or 3 days at 35°C. Exposure of eggs to 23.5°C or 50°C caused reduced infectivity. Hosts are infected by ingestion of embryonated ova. Worms reach maturity in 18 days after oral inoculation of chickens, but the prepatent period is 20–21 days. Pigeons experimentally infected with *C. obsignata* remained infected for about 9 months.

Pathogenicity

Birds heavily infected with *C. obsignata* tend to huddle, and may suffer emaciation, diarrhea, hemorrhagic enteritis, anorexia, lowered feed efficiency, fluid and metabolite losses, and sometimes death. A catarrhal exudate in the upper intestine and some thickening of the wall are seen in heavy infections (76). Experimentally, some weight depression was seen with as few as 14 worms (46); in other cases, infections of 100–1000 worms cause no weight changes. Poor feed conversion may result. Given the "wireworm" appearance of this nematode, it is very probable that it is thigmokinetic in the mucosa and may induce considerable villar detachment.

Experimentally infected chickens had no significant differences in total white blood cells or packed cell volume (76), although globulins and total protein may be increased (7). Conversely, in heavily infected pigeons, there was a marked decrease in total protein and albumen, as well as decreased plasma carotenoids and liver vitamin A (9).

Capillaria caudinflata *(Molin 1858) Wawilowa 1926, Capillariidae*

Hosts

C. caudinflata has been reported from chickens, turkeys, ducks, geese, guinea fowl, grouse, partridges, pheasants, pigeons, and quail. It is found in the mucosa of the small intestine.

Morphology

Male *C. caudinflata* are 9–18 mm long; the spicule is 0.7–1.2 mm long, tapering to a fine point distally; the spicule sheath has fine thornlike spines on the proximal portion; and there is a bursa present, supported dorsally by 2 T-shaped processes (Fig. 27.16C). Females are 12–25 mm long; the vulva has a characteristic appendage (Fig. 27.16D); and eggs are 47–58 × 20–24 μm, with a thick and finely sculptured shell.

Life Cycle

Earthworms of the species *Allolobophora caliginosa* or *Eisenia foetida* are intermediate hosts (2).

Capillaria bursata *Freitas and Almeida 1934, Capillariidae*

Hosts

C. bursata has been reported in chicken, turkey, goose, and pheasant.

Location

C. bursata may be found in the mucosa of the small intestine.

Morphology

The male is 11–20 mm long; spicules are 1.1–1.6 mm long; the sheath is without spines; and the bursa is round and supported by 2 dorsal and 2 ventral projections (Fig. 27.16E). The female is 16–35 mm long; the vulva has 2 semicircular valves (Fig. 27.16F); eggs are 51–62 × 22–24 μm, with a shell that has fine longitudinal ridges.

Life Cycle

Eggs are passed in the feces, and larval development is complete in 8–15 days. Eggs hatch after ingestion by earthworms, releasing larvae, which become infective to the final host after 22–25 days. Worms mature in the final host 20–26 days after ingestion.

Capillaria anatis *(Schrank 1790) Travassos 1915, Capillariidae*

Hosts

C. anatis has been reported in chicken, turkey, duck, goose, partridge, and pheasant.

Location

C. anatis usually is found in the cecum, sometimes in the small intestine.

Morphology

C. anatis are threadlike worms. The male is 8–15 mm long; the spicule is 0.7–1.9 mm long with a spiny sheath; and the tail has 2 lobes but no lateral caudal alae (Fig. 27.16G). The female is 11–28 mm long; the vulva is without appendage (Fig. 27.16H); and eggs are 46–67 × 22–29 μm with a thick rough outer shell.

Life Cycle

The life cycle of *C. anatis* is unknown.

Ornithostrongylus quadriradiatus *(Stevenson 1904) Travassos 1914, Heligmosomidae*

Hosts

O. quadriradiatus is found in the lumen of the small intestine of pigeons and doves.

Morphology

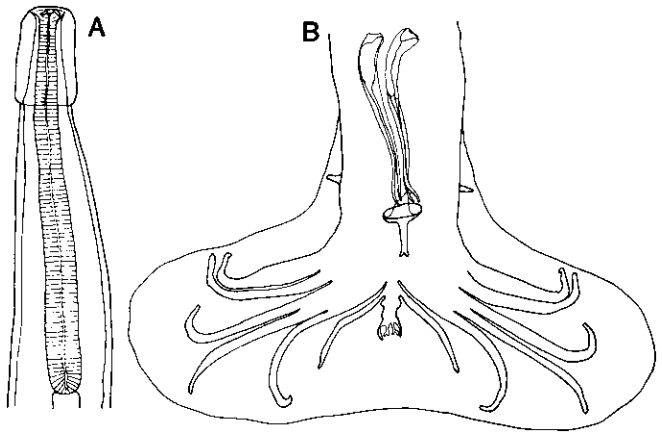
O. quadriradiatus worms are delicate, slender, and red when freshly collected, apparently from ingested blood in the intestine, and they have a cuticle about their head inflated to form vesicular enlargement (Fig. 27.18A). The male is 9 to 12 mm long; the bursa is bilobed, with no distinct dorsal lobe; the dorsal ray is much shorter than other rays, not extending halfway to the bursal margin, bifurcating near its tip to form 2 short tips; there is a stumpy process present on each side near the base of the ray; spicules are equal, 150–160 μm long, somewhat curved, each terminating in three pointed processes (Fig. 27.18B); and the telamon is 57–70 μm long, with 2 longitudinal processes extending backward and forward along the dorsal wall of cloaca and 2 lateral processes forming a partial ring through which the spicules protrude. The female is 18–24 mm long; the vulva is near the end of tail; the vagina is short, followed by 2 powerful muscular ovajectors; the tail tapers to a narrow blunt end, bearing a short spine; and eggs contain developing embryos when deposited.

Life Cycle

Mature worms are found in the small intestine. The oval, thin-shelled eggs are voided in the droppings and hatch in approximately 19–25 hours, depending on moisture and temperature. After hatching, the larva molts twice within the next 3 or 4 days to reach the infective stage. The infective larvae are swallowed by a pigeon or other susceptible host and mature in the small intestine. The female worm matures in 5–6 days after infection.

Pathogenicity

Pigeons may suffer catarrhal enteritis and blood loss. Heavily infected birds are moribund and anorexic. There is a pronounced greenish diarrhea, and the bird gradually wastes away. Signs of difficult and rapid breathing usually precede death. Intestines of fatally infected birds are markedly hemorrhagic and have a greenish mucoid content with masses of sloughed epithelium.



27.18. *Ornithostrongylus quadriradiatus*. A. Head. B. Bursa of male. (After Stevenson)

Deletrocephalus dimidiatus

Host

Rhea, small and large intestines.

Morphology

Worms are 11 to 24 mm in length. Spicules are approximately 1 mm long and eggs are 70 × 120 μm. Buccal capsule is prominent and armed with gutter teeth.

Life Cycle

Probably direct.

Pathogenesis

Hematophagic activity of this nematode leads to anemia.

Nematodes Found Primarily in the Cecum

Heterakis dispar *(Schrank 1790) Dujardin 1845, Heterakidae*

Hosts

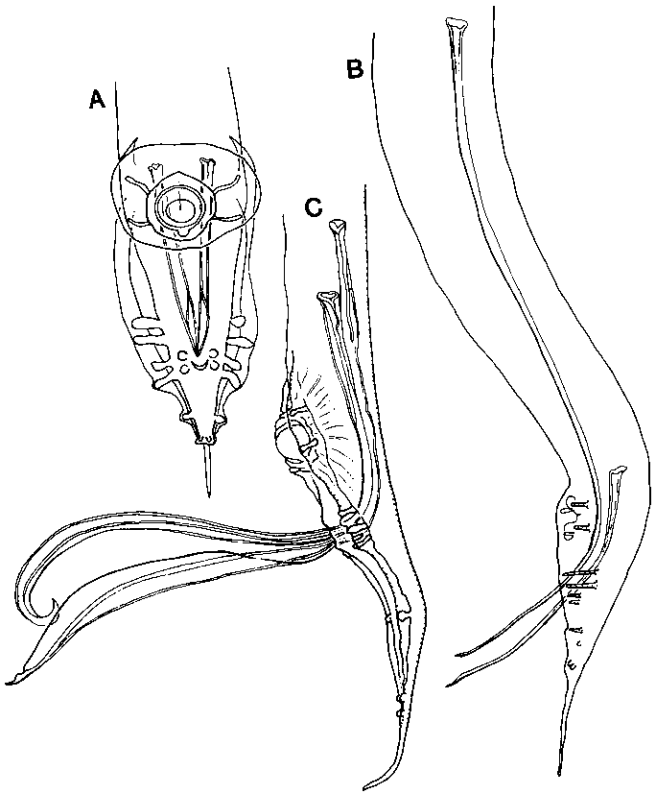
H. dispar infects the cecal lumen of ducks and geese.

Morphology

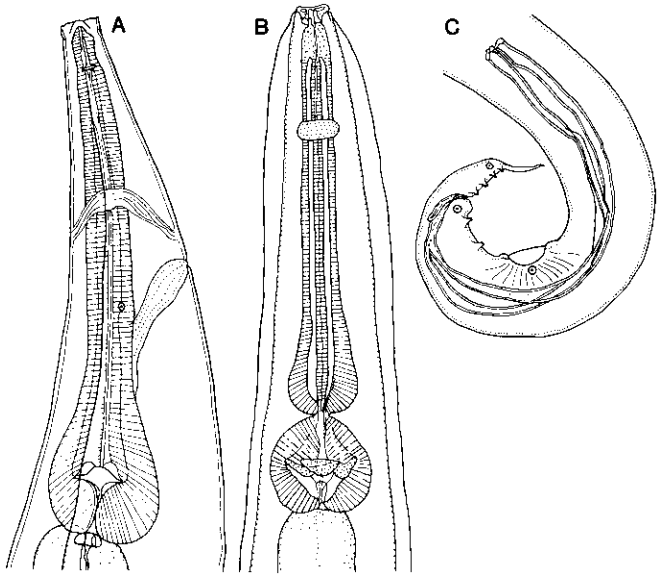
H. dispar is somewhat larger than *H. gallinarum* but similar in appearance except for spicules. The male is 7–18 mm long and has a preanal sucker 109–256 μm in diameter; its spicules are short and essentially equal, 390–730 μm long (Fig. 27.19A). The female is 16–23 mm long, and eggs are 59–62 × 39–41 μm.

Life Cycle

Direct, similar to *H. gallinarum.*



27.19. Male tails. A. *Heterakis dispar*. (After Madsen) B. *Heterakis gallinarum*. (After Lane) C. *Heterakis isolonche*. (After Cram *et al.*)



27.20. A. *Heterakis gallinarum*, head. B. *Subulura suctoria*, head. (After Skrjabin and Shikhobalova) C. *Subulura strongylina*, male tail. (After Barreto)

Pathogenicity

H. dispar is relatively nonpathogenic.

Heterakis gallinarum *(Schrank 1788) Madsen 1950, Heterakidae*

Hosts

H. gallinarum is found in the ceca of chickens, turkeys, ducks, geese, grouse, guinea fowl, partridges, pheasants, and quail. Along with *C. obsignata* and *A. galli*, this nematode is an extremely common and abundant parasite of replacement and productive commercial chickens that are maintained on ground or litter.

Morphology

Adult worms are small and white; the mouth is surrounded by three small, equal-sized lips; 2 narrow lateral membranes extend almost the entire length of the body; and the esophagus ends in a well-developed bulb containing a valvular apparatus (Fig. 27.20A). The male is 7–13 mm long; the tail is straight, ending in a subulate point; there are 2 large lateral bursal wings; the preanal sucker is well developed, with strongly chitinized walls and a small semicircular incision in the posterior margin of the sucker wall; there are 12 pairs of caudal papillae, the 2 most posterior pairs being stout and superimposed; and spicules are dissimilar, the right one being 0.85–2.8 mm long, and the left one being 0.3–1.1 mm long with a curved tip (Fig. 27.19B). The female is 10–15 mm long; the tail is long, narrow, and pointed; the vulva is not prominent and is slightly posterior to the middle of the body; and eggs are thick shelled, ellipsoidal, unsegmented when deposited, undistinguishable from those of *A. galli,* and 63–75 × 36–50 μm.

Life Cycle

The life cycle is direct. Adults in the ceca produce eggs which pass unembryonated in the feces. In approximately 2 weeks, eggs reach the infective stage. When swallowed by a susceptible host, the larvae hatch in the upper intestine and reach the ceca within 24 hrs. Larvae are closely associated with the cecal mucosal tissue until 12 days postinfection, when they become essentially free in the lumen. At necropsy, most of the adult worms are found in the blind ends of the ceca. Although not a normal part of the life cycle, eggs may be ingested by earthworms, where they hatch and live for months. Earthworms may later be ingested by birds, resulting in infections with cecal worms and also *Histomonas meleagridis,* which is carried by the cecal worm. The greatest susceptibility is with the ring-necked pheasant, followed by the guinea fowl and chicken (47). Commercial turkeys rarely develop patent infections, but larval parasitism is sufficient to allow the initiation of histomoniais (see under "Pathogenicity").

Pathogenicity

The ceca show marked inflammation and thickening of the walls. In heavy infections, nodules form in the mucosa and submucosa, as the response of already sensitized ceca to subsequent infection (41). Hepatic granulomas containing the worms have also been reported in chickens (61).

The chief importance of the cecal worm lies in its role as a carrier of the blackhead organism *Histomonas meleagridis.* Blackhead may be produced in susceptible birds by feeding embry-

onated eggs of *H. gallinarum* taken from blackhead-infected birds. Most isolates of heterakid ova are positive for *H. meleagridis.* Histomonads were found incorporated in the worm egg (74), and their presence was identified in the gut wall, in the reproductive systems of the male and female, and in the developing eggs of this cecal worm (31). Direct transmission of *Histomonas meleagridis* was accomplished using larvae (63) and male worms (67).

Turkeys are highly susceptible to blackhead disease. Commercial turkeys do not support patent infections of *H. gallinarum,* hence, they do not pass eggs of this nematode; eggs that were previously thought required by *Histomonas meleagridis* for survival in the environment and eventual passage of the protozoan to other turkeys. In a series of studies conducted by McDougald and associates, a most probable means of progression of histomoniais through turkey flocks has been elucidated (36, 49). After the initial, accidental infection of a few turkeys in a flock (larvated *Heterakis* eggs brought into a flock on contaminated boots, equipment, etc), histomonad proliferation in the ceca of the turkeys immediately ensues, and histomonad stages are expelled in the very watery droppings of these infected birds and immediately inoculated into flock mates by their "cloacal drinking". The above, experimentally-proven epidemiological events provide a scenario for the rapid transmission of Blackhead disease in turkey flocks, even with the lack of patent or sustained larval *Heterakis* infections in commercial turkeys. At present, given the lack of effective histomonacides on the market, the most effective means of controlling Blackhead in turkeys is to prevent the initial, accidental infection. In addition, McDougald advises the use of in-house barriers to turkey movement, especially around entry points; this, to minimize the spread of blackhead in a turkey barn once an infection has been started.

Not all isolates of *H. gallinarum* eggs are capable of inducing Blackhead disease in turkeys. In one set of studies, 10 different isolates of eggs were gathered from cecal worms obtained from spent breeder hens, larvated, and given to groups of 3-week-old turkeys. Mortality rates of the recipient turkeys varied from 0 to 50% (84).

Heterakis isolonche *Linstow 1906, Heterakidae*

Hosts

H. isolonche has been reported in duck, grouse, pheasant, prairie chicken, and quail. It is found in the lumen of the cecum or mucosa; larvae are found in the mucosa.

Morphology

H. isolonche is similar to *H. gallinarum* but easily differentiated based on the spicules. The male is 5.9–15 mm long; it has a preanal sucker, 70–150 μm in diameter; and spicules are long and essentially equal, 0.72–2.33 mm (generally 1.4–1.9 mm) long (Fig. 27.19C). The female is 9–12 mm long, and eggs are 65–75 × 37–46 μm.

Life Cycle

The life cycle is direct, similar to that of *H. gallinarum* but with a more extensive tissue phase. The second-stage larvae mature in the cecal mucosa, where adults may also be found.

Pathogenicity

H. isolonche reportedly caused mortality in pen-reared pheasants exceeding 50%. Diarrhea and weight depression are common. The invasion of the mucosa causes lymphocyte infiltration and granulation that leads to the formation of nodules in the cecal wall. These nodules may coalesce to form a thickened wall. In quail and grouse, there is little pathology, even when worms are present in large numbers.

Subulura brumpti *(Lopez-Neyra 1922) Cram 1926, Subuluridae*

Hosts

S. brumpti has been reported in chicken, turkey, dove, duck, grouse, guinea fowl, partridge, pheasant, and quail.

Location

Adult *S. brumpti* are found in the lumen of the cecum.

Morphology

S. brumpti are small nematodes with the anterior end curved dorsally; the mouth is hexagonal, surrounded by six weakly developed lips, each with median papillae; there are 2 pairs of larger papillae located dorsally and ventrally, well-developed amphids laterally, and anterior portions of the esophageal wall that are cuticularized, forming three teeth-like structures; the esophagus is dilated posteriorly, followed by a bulb (Fig. 27.20B); and there are cephalic alae extending to the anterior portion of intestine. The male is 6.9–10 mm long and 340–420 μm wide; the esophagus is 0.98–1.1 mm long; the tail is curved ventrally and ends in prolongation; there are caudal papillae (10 pairs) consisting of 3 pairs preanal, 2 pairs adanal, and 5 pairs postanal; caudal alae are narrow and not well developed; the preanal sucker is 170–220 μm long; spicules are similar and equal, 1.22–1.5 mm long; and the gubernaculum is 150–210 μm long. The female is 9–13.7 mm long and 460–560 μm wide; the esophagus is 1–1.3 mm long; the tail is straight and conical, ending in a sharp point; the vulva is anterior to the middle of the body; eggs are almost spherical, thin-shelled, 82–86 × 66–76 μm, and fully embryonated when laid.

Life Cycle

Embryonated ova pass from definitive hosts in cecal droppings. Larvae hatch in 4–5 hours and are consumed by beetles or cockroaches (4, 18). Larvae develop in the body cavity of the insect to the third, or infective stage. When the definitive host swallows an infected insect, the larvae migrate to the ceca and develop to the fourth stage within about 2 weeks. The final molt takes place on about the 18th day after infection. Patency is reach within about 6 weeks after infection.

Pathogenicity

No noticeable lesions were produced by this worm in the ceca of the quail (16). The cecum showed no evidence of inflammatory reactions, even though infection could persist as long as 8 months (18).

Subulura strongylina *(Rudolphi 1819) Railliet and Henry 1912, Subuluridae*

Hosts

S. strongylina has been reported in chicken, guinea fowl, and quail.

Location

S. strongylina is found in the lumen of the cecum.

Morphology

The lateral cephalic alae are well developed and extend from head to the median part of the esophageal bulb. The male is 4.4–12 mm long; the tail is curved into a V or an O shape; the preanal sucker is long and slender, 169 μm long; there are 11 pairs of caudal papillae; and spicules are equal, 890 μm to 1.2 mm long (Fig. 27.20C). The female is 5.6–18 mm long; the vulva is slightly anterior to the middle of the body; and eggs are 84 × 67 μm and embryonated when deposited.

Life Cycle

The exact life cycle of *S. strongylina* is unknown.

Pathogenicity

No noticeable lesions are produced in the ceca of quail.

Subulura suctoria *(Molin 1860) Railliet and Henry 1912, Subuluridae*

Hosts

Chickens, turkeys, guinea fowl, partridges, pheasants, and quail are hosts. It is found in the lumen or mucosa of the ceca or small intestine.

Morphology

This worm is larger than *S. brumpti.* The lateral cephalic alae are small and extend to the middle of the esophagus. The male is 11.8–13.8 mm long; spicules are equal and curved, 1–1.5 mm long. The female is 20–33 mm long, and eggs are 51–70 × 45–64 μm.

Life Cycle

The life cycle of *S. suctoria* is similar to that of *S. brumpti.* Beetles serve as intermediate hosts.

Pathogenicity

Barus and Blazek (6) reported little pathology.

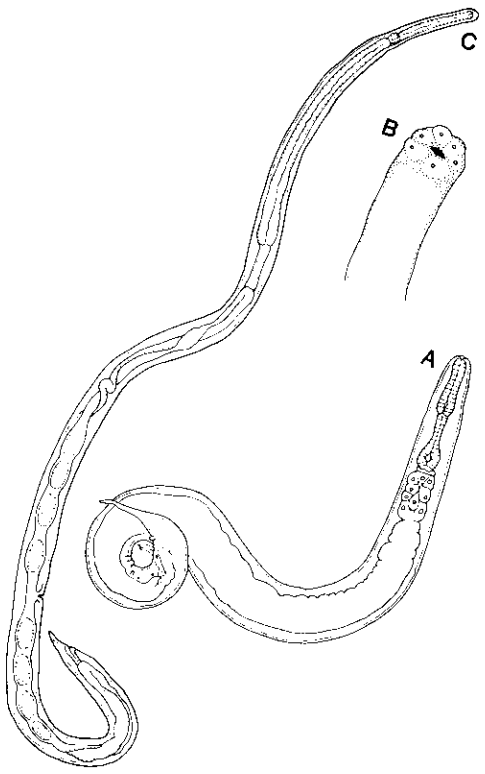
Strongyloides avium *Cram 1929, Strongyloididae*

Hosts

S. avium infects chickens, turkeys, geese, grouse, and quail. This extremely small roundworm has been reported from chickens in Puerto Rico (13), the junco (*Junco hyemalis*) in Virginia, and the coot (*Fulica americana*) in North Carolina. It is found in the cecum, sometimes in the small intestine.

Morphology

S. avium is characterized by a parasitic generation consisting of only parthenogenetic females in the intestine of the avian host,



27.21. *Strongyloides avium*. A. Free-living male. (After Cram) B. Head, parasitic female. C. Parasitic (parthenogenetic) female. (B and C after Sakamoto and Sarashina)

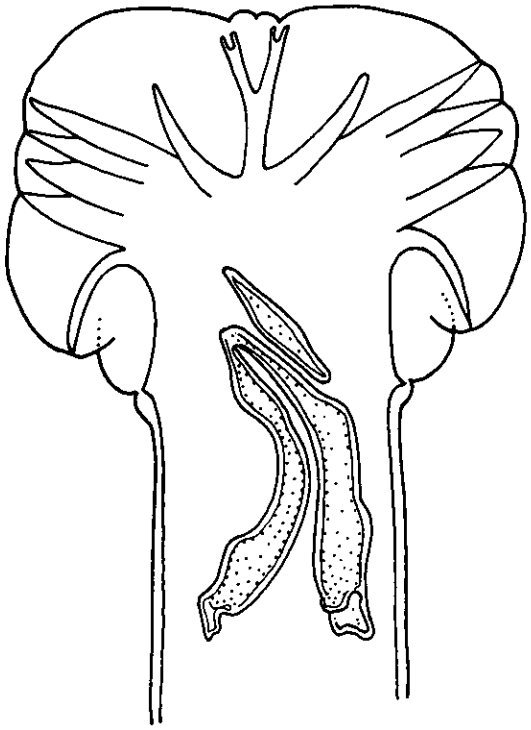
and a free-living generation of both males and females in the environment (Fig. 27.21A). The parasitic adult female is 2.2 mm long and only 40–45 μm wide; the vulva has projecting lips and is located 1.4 mm from the head end (Fig. 27.21B and C); the uteri are divergent from the vulva; ovaries are recurrent with simple "hairpin bends", the esophagus is noticeably elongate, and eggs have very thin shells, segmenting when deposited, and are 52–56 × 36–40 μm.

Life Cycle

Unlike most species of nematodes, the parasitic cycle of *S. avium* consists of females only. Eggs hatch soon after being passed in the droppings, sometimes in as soon as 18 hours. Larvae develop in the soil to free-living adult males and females (heterogonic life cycle), or infective larvae (homogonic life cycle). Progeny of the free-living generation are infective, "filariform" larvae that develop into parthenogenetic females after being swallowed by a susceptible host. Infection of the host through the skin is also possible. Infective larvae of this genus can invade the skin of humans and cause cutaneous eruptions (larva currens), which is of some medical concern.

Pathogenicity

The walls of the ceca are greatly thickened; typical pasty cecal contents almost disappear, and cecal discharges are thin and



27.22. *Trichostrongylus tenuis*. Bursa of male. (After Railliet)

bloody. If the fowl survives the acute stage, the ceca gradually regain function and the thickening of the walls decreases. Young birds suffer most from infections. Light infections cause little clinical effect.

Trichostrongylus tenuis *Mehlis 1846, Trichostrongylidae*

Hosts

T. tenuis infects chickens, turkeys, ducks, geese, guinea fowl, pigeons, emu, and quail. It is found in the ceca and sometimes in the small intestine.

Morphology

T. tenuis worms are small and slender; the body gradually attenuates in front of the genital opening; the mouth is surrounded by three small, inconspicuous lips; the cuticle at the anterior end of the body is lacking in conspicuous striations for a distance of about 200–250 μm from the extremity. The male is 5.5–9 mm long and 48 μm wide near the center of the body; the cuticle is inflated on the ventral surface just anterior to the bursa; the bursa has 1 dorsal and 2 lateral lobes, the dorsal one not distinctly marked off from the lateral; each lateral lobe is supported by 6 rays (Fig. 27.22); the dorsal ray bifurcates at its distal third, and each of these divisions again bifurcates and is very finely pointed; spicules are dark brown and slightly unequal in length, the longer being 120–164 μm, and the shorter being 104–150 μm; both are twisted, especially at distal ends, and have an ear-like structure on the proximal end. The female is 6.5–11 mm long and 77–100 μm wide at the level of the vulva; the vulva is in the posterior end of the body, with crenulated edges; uteri are divergent; and eggs are thin shelled.

Life Cycle

This worm has a direct life cycle. *T. tenuis* from pheasants has been transmitted successfully to domestic turkey and guinea fowl. Chickens have been experimentally infected (77). Eggs hatch within 36–48 hours in the droppings, and the larvae molt twice to become infective in approximately 2 weeks. When picked up by a susceptible host, the larvae molt twice more in the ceca of the bird before becoming adults.

Pathogenicity

T. tenuis was associated with decimation of the red grouse population in Scotland. A fatal dose can be as low as 500 infective larvae. Ceca become extended, and blood vessels show congestion. The mucosa of the ceca is inflamed, and the ridges are greatly thickened. Severe infection causes weight loss and anemia. *T. tenuis* can also be fatal to young goslings. Heavy mortality occurs usually in the fall, mainly in the young birds in that year's hatching, and again in the spring. These 2 seasons are not isolated epidemics, but rather are the peaks of a disease that continues in a chronic form the entire year. With emus, a bloody, mucoid diarrhea is extensive. There is no protective immunity due to prior infection.

Aulonocephalus lindquisti *Chandler 1934, Subuluridae*

Host

A. lindquisti have been reported in bobwhite quail and blue or scaled quail, mainly in western Texas.

Location

A. lindquisti is most commonly found in the cecum, although sometimes in the large intestine.

Morphology

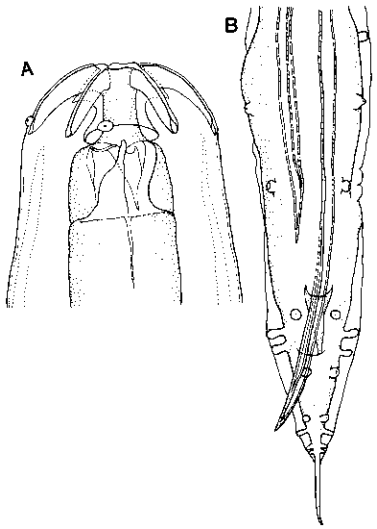
A. lindquisti are bright pink worms; the cuticle is finely striated; cervical alae are present, 45–65 μm in breadth in the female but only 20–25 μm in breadth in the male; the head has 6 trough-like grooves about 65–70 μm long radiating from the mouth (Fig. 27.23A); the esophagus is club shaped, 1.3–1.8 mm long, with the bulb slightly longer than it is broad. The male is 8–10.6 mm long and 420–490 μm wide; the gubernaculum is 170–190 μm long; spicules are approximately equal, 1.16–1.3 mm long (Fig. 27.23B). The female is 10–14.8 mm long and 530–590 μm wide; the vulva is inconspicuous, ranging from slightly anterior to slightly posterior to the middle of the body; the tail terminates in a thin spike; and eggs are broadly oval, 58 × 42–45 μm.

Life Cycle

The life cycle of *A. lindquisti* is unknown.

Pathogenicity

The pathologic effects of this species are unknown, but as many as 300 worms have been recovered from a single host.



27.23. *Aulonocephalus lindquisti*. A. Head. B. Male tail. (After Chandler)

Codiostomum struthionis

Host
Ostrich.

Location
Cecum and colon.

Morphology
Large buccal capsule with both external and internal leaf crowns.

Life Cycle
Unknown, but most likely direct.

Pathogenesis
Not determined.

Other Nematodes of the Digestive Tract

Numerous species of nematodes have been found in domestic poultry in other parts of the world. Occasionally, some are reported from North America from imported birds. The following are some of these species.

Esophagus and Crop

These include *Gongylonema crami* from chickens in Java, *G. congolense* from chickens and ducks in Africa and *G. sumani* from chickens in India. *Capillaria cairinae* is found in the esophagus of ducks in Brazil, and *C. combologiodes* is found in the crop of turkeys in Europe. Larvae of *Spirocerca lupi* have been found encysted in the crop of chickens in the southern United States.

Proventriculus

Parhadjelia neglecta (Habronematide) has been reported in domestic ducks in Brazil. *Echinuria jugadornata* was reported from the former Soviet Union. *Physaloptera acuticauda* was reported from chickens and pheasants in Brazil and from falconiform birds in the United States. *Tetrameres* species were reported from various birds, including *T. confusa* from chickens, turkeys, and pigeons in South America and Asia; *T. gigas* from domestic ducks in South America; *T. mohtedae* from chickens in India; and *T. spinosa* from chickens and domestic ducks in India.

Gizzard

Several nematodes not described above are found under the gizzard lining of domestic poultry. *Histiocephalus laticaudatus* has been recovered from chickens and ducks and *Streptocara pectinifera* from chickens and guinea fowl in Europe. *Epomidiostomum orispinum* is found in domestic ducks and geese in Europe and Africa, and *E. skrjabini* is found in domestic geese in Asia.

Small Intestine

Abbreviata gemina, a *Physaloptera*-like worm, occurs in chickens in Egypt. The anisakid *Contracaecum microcephalum* infects domestic ducks in Europe, Asia, and Africa, and *Porrocaecum crassum* is in domestic ducks and guinea fowl in Europe. *Capillaria anseris* occurs in domestic geese in Europe. *Hartertia gallinarum*, which uses a termite as an intermediate host, causes diarrhea and decreased growth and egg production in chickens in Africa.

Cecum

Numerous species of *Heterakis* not mentioned above are found in chickens throughout the world. These include *H. beramporia*, Asia; *H. bervispiculum*, South America and Africa; *H. caudabrevis*, former Soviet Union; *H. indica*, India; and *H. linganensis*, China. Turkeys in China are infected with *H. meleagris*. *Subulura differens* is widespread in chickens, guinea fowl, and quail in South America, Europe, Africa, and Asia and sometimes is found in the small intestine. Several *Capillaria* have been described including *C. monteividensis* and *C. uruguayensis* from chickens in Uruguay and *C. spinulosa* from ducks in Europe.

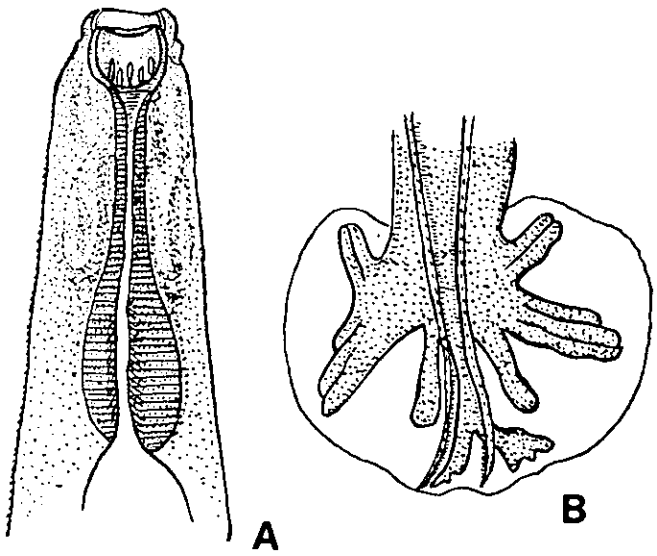
Nematodes of the Respiratory Tract

Cyathostoma bronchialis *(Muehlig 1884) Chapin 1925, Syngamiidae*

Hosts
C. bronchialis has been reported in ducks, geese, and turkeys (and chickens, experimentally). *C. variegatum* has been reported from the emu in the United States and is extremely similar in all aspects to *C. bronchialis*.

Location
C. bronchialis is found in the larynx, trachea, bronchi, and sometimes in the abdominal air sacs.

Morphology
C. bronchialis is very similar to *Syngamus* but is larger and less firmly united *in copula*; the buccal capsule is somewhat wider than



27.24. *Cyathostoma bronchialis*. A. Head. B. Male tail.

deep, with usually 6 but occasionally 7 triangular buccal teeth (Fig. 27.24A). The male is 8–12 mm long and 200–600 μm wide; spicules are long and slender, 540–870 μm, with tips slightly curved inward (Fig. 27.24B). The female is 16–30 mm long, 750 μm–1.5 mm wide; the vulva has fairly prominent lips, situated in the terminal portion of the anterior third of the body; the tail is acute; and eggs are 68–90 × 43–60 μm, with slight opercula.

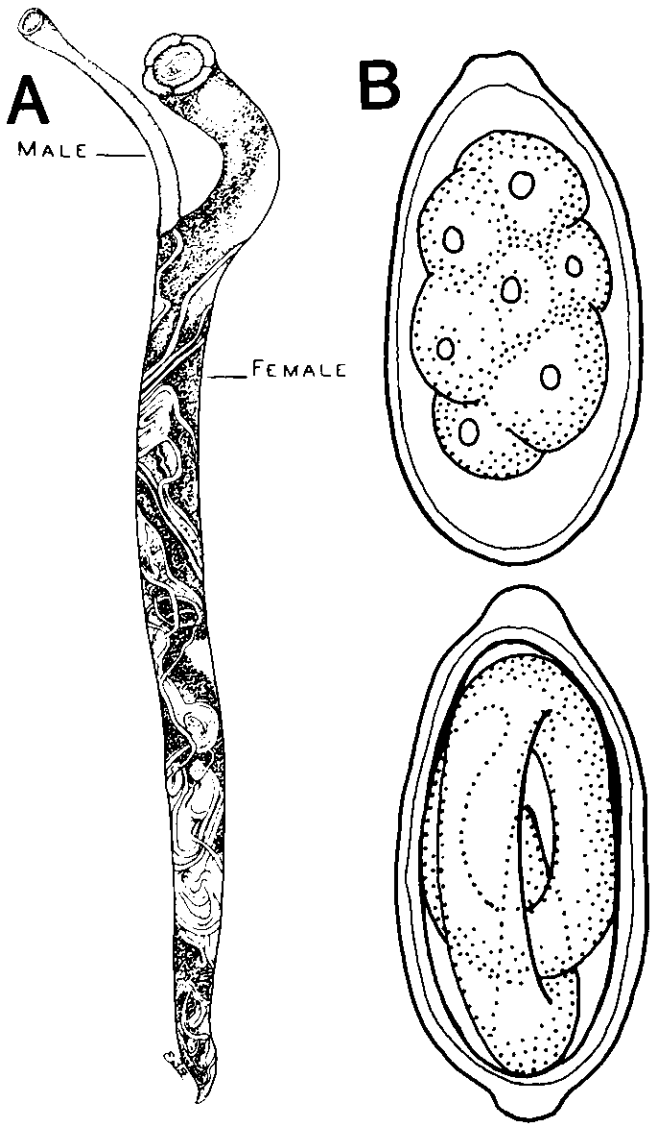
Life Cycle

The life cycle of this species of gapeworm may be direct or indirect. Infections may begin directly with ingestion of third-stage larvae, or indirectly by the ingestion of a paratenic host (earthworms). The infecting third-stage larvae migrate to the lungs through the peritoneal cavity and air sacs, rather than through the bloodstream as does *S. trachea* (29). The larvae molt twice in the lung at 1 and 4 days post-infection. They migrate into the trachea at 6 days, copulate at 7 days, and reach full maturity by 13 days post-infection. Eggs are first found in the tracheal mucus 13 days post-infection.

Pathogenicity

Morbidity of 80% with a mortality of 20% was reported in a flock of domestic geese near Duluth, Minnesota (33). The course of the disease lasted 5 months, during which time the birds showed signs of respiratory distress (gaping). Severely affected birds died soon after the appearance of respiratory disturbances and signs may be similar to those of laryngotracheitis. Recovered birds showed growth retardation.

Experimentally infected domestic geese developed bronchitis of the primary, secondary, and tertiary bronchi (30). During prepatency, hyperplasia of the epithelium of the primary bronchi was the predominant lesion. During patency, generalized pneumonitis was prominent in response to aspirated nematode eggs. Mandarin ducks, infected via earthworms, suffered dyspnea and



27.25. *Syngamus trachea*. A. Male and female worms. (After Wehr) B. Egg.

mortality (87). Other earthworms from the same source were found to harbor 4–5 nematode larvae each.

Syngamus trachea *(Montagu 1811) Chapin 1925, Syngamidae*

Host

S. trachea has been reported in chicken, turkey, goose, guinea fowl, pheasant, peafowl, emu, and quail. It is found in the trachea, bronchi, and bronchioles.

Morphology

S. trachea are called "redworms" because of their prominent color, "forked worms" because the male and female are always locked in copulation to form a "Y" (Fig. 27.25A), and "gapeworms", because birds tend to gasp or "gape" with heavy infection. *S. trachea* has an orbicular mouth, with a hemispheric

chitinous capsule, usually with 8 sharp teeth at the base; the mouth is surrounded by a chitinous plate, the outer margin of which is incised to form 6 festoons opposite each other. The male is 2–6 mm long; the bursa is obliquely truncated and is provided with rays, sometimes with strikingly asymmetrical dorsal rays; spicules are equal, slender, short, and 57–64 μm long. The female is 5–20 mm long (longer in the turkey); the tail end is conical, bearing a pointed process; the vulva is prominent, about one-fourth of the body length from anterior end, but the position varies with age; and eggs are 90 × 49 μm, ellipsoidal, and operculated (Fig. 27.25B).

Life Cycle

Transmission from bird to bird may be either direct (by the ingestion of embryonated eggs or infective larvae) or indirect (by ingestion of earthworms or other invertebrates containing free or encysted gapeworm larvae). The female gapeworm deposits eggs through the vulvar opening underneath the bursa of the attached male into the lumen of the trachea. The eggs reach the bird's mouth cavity, are swallowed, and pass to the outside in the droppings. Eggs embryonate and hatch in about 8–14 days, and larvae live free in the soil for a time. The earthworms *Eisenia foetidus* and *Allolobophora caliginosus* become infected with gapeworm larvae, where the larvae enter the body cavity and invade the body musculature and encyst. Gapeworm larvae in the earthworm remain infective to young chickens for as long as 4 years. Slugs and snails may also serve as transfer or auxiliary hosts of larvae. While the paratenic host is not necessary for transfer of gapeworms to other birds experimental infection has been more successful by feeding infected earthworms.

Infective larvae either penetrate the wall of the crop and esophagus and migrate to the lungs or penetrate the duodenum and are carried to the lungs by the portal bloodstream (5, 28). Molting and development to the adult stage occur in 4–5 days and worms produce eggs in about 2 weeks. The importance of wild birds in the spread of gapeworm disease to poultry is still unclear.

Pathogenicity

In the United States, *S. trachea* is the causative agent of "gapes" (labored breathing due to parasites) in chickens, turkeys, peacocks, emus, and pheasants (Fig. 27.26).

Gapeworms represent a serious menace to pheasant production in the United States. Confinement rearing of young chickens has reduced outbreaks, but this parasite continues to present problems for turkeys raised on range.

Young birds are the most seriously affected by gapeworms. The rapidly growing worms soon obstruct the lumen of the trachea and cause the birds to suffocate. Turkey poults, baby chicks, and pheasant chicks are most susceptible to infection. Turkey poults usually develop gapeworm signs earlier and begin to die sooner after gapeworm infection than do young chickens. Experimentally infected guinea fowls, pigeons, and ducks do not exhibit characteristic signs of gapeworm infections. Adult birds rarely develop heavy infections.

The trachea of infected birds becomes irritated with inflamed mucous membranes, resulting in coughing. Lesions are usually found in the trachea of turkeys and pheasants but seldom in the trachea of young chickens and guinea fowl. These lesions or nodules result from an inflammatory reaction at the site of permanent attachment of the male worms. The female worms apparently detach and reattach. The net blood loss with *S. trachea* is minimal. Turkey poults may have marked heterophilia, monocytosis, eosinophilia, lymphocytopenia, and a decreased packed cell volume (37).



27.26. *Syngamus trachea*. Trachea showing attached gapeworms. (After Wehr)

Nematodes of the Eye and Associated Structures

Of 70 known species of *Oxyspirura*, only 3 (*O. mansoni, O. petrowi,* and *O. pusillae)* have been reported from North America north of Mexico. *O. petrowi* is a species of wide geographic range. It shows little host specificity and has been found in 14 species of wild birds in Louisiana and 5 species in Michigan. Although this species has not been reported in domestic chickens, it is found in grouse and prairie chickens.

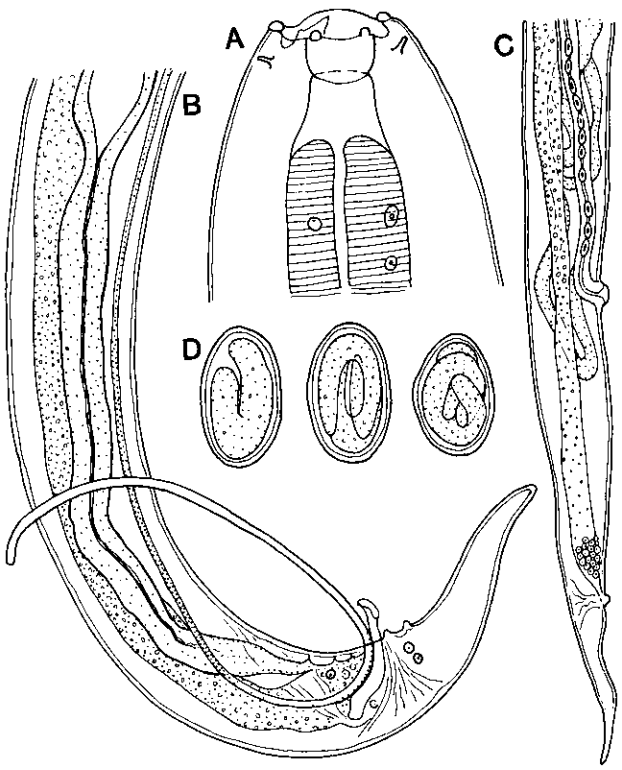
Oxyspirura mansoni *Cobbold 1879, Thelaziidae*

Hosts

O. mansoni has been reported in chickens, turkeys, ducks, grouse, guinea fowl, peafowl, pigeons, and quail. It is located beneath the nictitating membrane, and in the conjunctival sacs and nasolacrimal ducts.

Morphology

The body of *O. mansoni* is attenuated at both ends, with the anterior rounded and the posterior pointed; the cuticle is smooth; no membranous appendages exist; the mouth is circular, surrounded



27.27. *Oxyspirura mansoni*. A. Head. B. Male tail. C. Female tail. D. Eggs. (B–D after Ransom)

by a 6-lobed chitinous ring with 2 lateral and 4 submedian papillae in relation to the clefts of this ring; 2 pairs of subdorsal and 1 pair of subventral teeth are in the mouth cavity; the buccal cavity has a short, wide anterior portion and a long, narrow posterior portion (Fig. 27.27A). The male is 8.2–16 mm long and 350 μm wide; the tail is curved ventrally, without alae; there are 4 pairs of preanal and 2 pairs of postanal papillae; spicules are unequal (Fig. 27.27B), one 3–4.55 mm long, and the other, 180–240 μm. The female is 12–20 mm long and 270–430 μm wide; the vulva is 0.78–1.55 mm wide; the anus is 400–530 μm from the tip of the tail (Fig. 27.27C); and eggs are embryonated when deposited, 50–65 × 45 μm (Fig. 27.27D).

Life Cycle

Eggs of the mature worm are deposited in the eyes of the bird host, washed down the tear ducts, swallowed, and passed in the droppings. The cockroach *Pycnoscelus* (*Leucophaea*) *surinamensis* ingests the nematode eggs in the feces. Within approximately 50 days, the body cavity of the cockroach contains larvae infective to bird hosts. These larvae are often contained within cysts deep in the adipose tissue or along the course of the alimentary tract of the insect; sometimes larvae are free in the body cavity and legs of the cockroach. Upon ingestion by a susceptible host, the infective larva is freed in the crop, migrates up the esophagus to the mouth and through the nasolacrimal duct to the eye.

Various wild birds may serve as reservoirs of infection for poultry. The blackbird (*Agelaius phoeniceus*), bobolink (*Dolichonyx oryzivorus*), wild pigeon (*Columbia livia*), loggerhead shrike (*Lanius ludovicianus*), and blue jay (*Aphelocoma cyanea*) have been experimentally infected. The eyeworm occurs naturally in the English sparrow, mynah, Chinese dove, Japanese quail, and pheasant (*Phasianus torquatus torquatus* and *P. versicolor versicolor*) in Hawaii. In Hawaii, the local wild birds appear to be of little importance in the dissemination of this poultry parasite (65).

Pathogenicity

Infected birds show a peculiar ophthalmia and may scratch at the eyes. The nictitating membrane becomes swollen, projects slightly beyond the eyelids at the corners of the eyes, and usually is kept in continual motion, as if trying to remove some foreign object from the eye. The eyelids sometimes become stuck together, and a white cheesy material collects beneath them. If left untreated, severe ophthalmia may develop; as a result, the eyeball may be destroyed. The worms are rarely found in the eyes when severe signs are manifested.

Oxyspirura petrowi *Skryjabin 1929, Thelaziidae*

Hosts

O. petrowi has been reported in grouse, pheasants, and prairie chickens.

Location

O. petrowi may be found beneath the nictitating membrane of the eye.

Morphology

The body of *O. petrowi* is slender, yellow to cream colored, bluntly rounded anteriorly, and attenuated posteriorly; cervical alae are present, with the cuticle transversely striated; the mouth has 4 submedian pairs and 3 circumoral pairs of cephalic papillae; an undivided cuticularized bursal capsule exists. The male is 6.3–8.6 mm long and 185–330 μm wide; the right spicule is 121–320 μm long and slender with a sharp tip. The female is 7.7–12.3 mm long and 200–455 μm wide; the vulva is 500–700 μm from the tip of the tail; the anus is 242–400 μm from the posterior extremity; and eggs are embryonated and 35–44 μm × 15–31 μm.

Life Cycle

The life cycle is similar to that of *O. mansoni*.

Pathogenesis

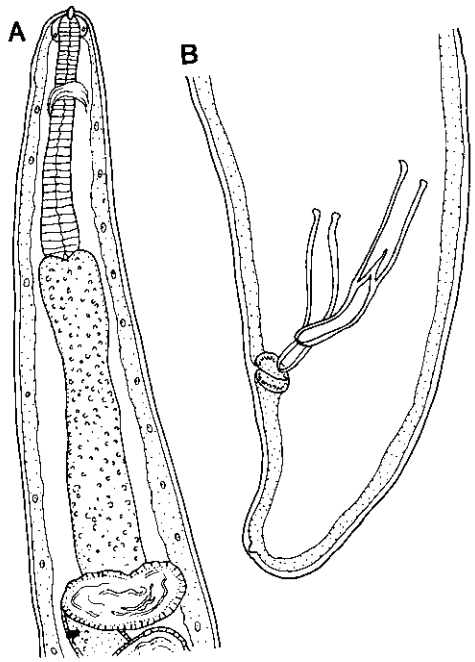
Infection with *O. petrowi* produces a condition similar to that seen with *O. mansoni.*

Tissue-Dwelling Nematodes Outside the Intestinal Tract

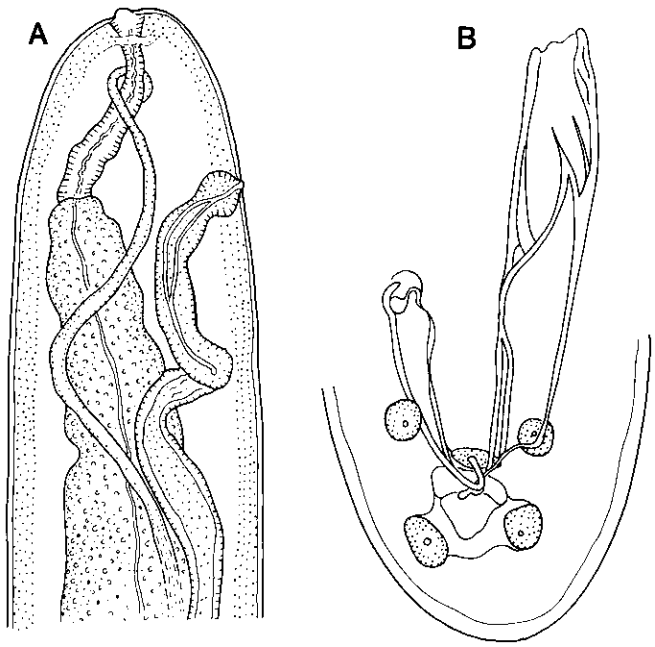
Aproctella stoddardi *Cram 1931, Dipetalonematidae*

Hosts

A. stoddardi is found in the body cavity of turkeys, doves, and quail. This species has been recovered from the bobwhite quail in the southern United States and from grouse in New England.



27.28. *Aproctella stoddardi*. A. Head. B. Male tail. (After Anderson)



27.29. *Singhfilaria hayesi*. A. Head. B. Mail tail. (After Anderson and Prestwood)

Morphology

The body of *A. stoddardi* is slender; the cuticle is divided into 4 fields, 2 medians longitudinally striated and 2 smooth laterals; the mouth is simple without definite lips (Fig. 27.28A). The male is 6–7.6 mm long and 60–140 μm wide; the spicules are stout and curved, with the right one 50–60 μm, and the left 73–90 μm (Fig. 27.28B). Caudal papillae are absent. The female is 13–16.5 mm long and 71–260 μm wide; the vulva is 1.3–1.6 mm from the anterior end, with no protuberance; the anus is 140–180 μm from the caudal extremity; there are no eggs; and unsheathed larvae are present in the uteri.

Life Cycle

The life cycle is unknown, but a biting arthropod is thought to be the intermediate host.

Pathogenicity

Small numbers of *A. stoddardi* are not pathogenic; however, heavy infection may result in mortality in doves. A granulomatous pericarditis has also been reported.

Singhfilaria hayesi *Anderson and Prestwood 1969, Onchocercidae*

Host

S. hayesi is found in subcutaneous tissues in the region of the esophagus, crop, and trachea of turkeys and quail in the southern United States.

Morphology

S. hayesi have no structures on the head (Fig. 27.29A); the cuticle has innumerable tiny, transverse thickenings. The male is 13.6 mm long and 250 μm wide; spicules are markedly dissimilar, with the right one tooth shaped, 81 um long, and the left 125 μm long, divided into a broad shaft and short filament (Fig. 27.29B). The anus is subterminal, 28 μm from the caudal extremity; the caudal papillae consist of 1 large pair of postanal papillae and 1 large medial papilla anterior to the anus. The female is 35–40 mm long and 420–500 μm wide; the vulva is 390–400 μm from the cephalic extremity; and there are microfilariae in uteri.

Life Cycle

The life cycle of *S. hayesi* is unknown.

Pathogenicity

Few pathologic lesions have been found.

Dicheilonema rhea

Dicheilonema rhea is a filariid of rheas. The nematode is 65 cm in length, approximately 3 mm wide, and is found in the abdominal and appendage fascia. Little pathology has been associated with this worm. The life cycle is unknown.

Other Tissue-Dwelling Nematodes

The guinea worm *Avioserpens taiwana* causes fibrous tumors in the subcutaneous tissue under the mandible and on the thigh of domestic ducks in Asia.

Fifteen genera of filarial nematodes that infect birds have been recognized (3). Many species are not host specific. *Cardiofilaria pavlovsky* and *Aproctella stoddardi* have been reported from at least 7 families of birds. In general, filarial nematodes cause little problem in domestic poultry. The species shown in Table 27.1

are from game birds in North America. Other species that infect chickens but have not been reported in North America include the following from India: *Aprocta babamii* from the heart, *Cardiofilaria mhowensis* from the body cavity, and experimentally-induced *Chandlerella quiscali*.

Chandlerella quiscali is of major importance in emus, particularly those that are less than 1 year in age. This nematode is commonly found in the brain ventricles of grackles, cowbirds, blue birds etc., but do not appear to cause any pathologic condition. In the emu, torticollis, incoordination, emaciation and eventual death are associated with cerebral infections. The primary intermediate host is *Culicoides crepuscularis,* but other hematophagic arthropods could also serve as intermediate hosts. Emus do not develop patent infections and hence, no microfilariemia.

Baylisascaris procynois and *Baylisascaris columnaris* from raccoons and skunks, respectively, are reported to cause avian cerebral and neural/spinal infections in a variety of birds including chickens, bush turkeys, partridges, emu, and quail. Affected birds often show high morbidity and mortality after consuming raccoon feces. The damage is caused by migrating larvae, which never reach adulthood in birds. This disease has been produced experimentally in chickens (42).

Prevention and Control

Modern poultry practices, such as the confinement rearing of broilers and pullets and caging of laying hens, have significantly decreased the quantity and variety of nematode infections in poultry. Many that cause extensive problems in backyard or farmyard flocks are seldom seen in commercial operations. Others, such as *Ascaridia, Capillaria*, and *Heterakis,* are still found in large numbers and of high incidence in all types of commercial bird production. In addition, increased pen-rearing of game birds has led to increased nematode problems in these species. At present, an increasing trend has been emerged for "organic" and / or "humane" bird maintenance and production wherein birds are placed in more natural settings without the prophylactic use of drugs (parasiticides, antibiotics, etc). This trend may lead to greater parasite incidence at a time when chemical intervention is decreased. This trend has already been recognized in the EU (25).

For nematode parasites, control measures should include sanitation, interruption of the life cycle(s), and strategic use of efficacious parasiticides. Confinement rearing on litter largely prevents infections with nematodes using intermediate hosts such as earthworms or grasshoppers. Conversely, nematodes with direct life cycles or those that use indoor intermediate hosts such as beetles may prosper. Treatment of the soil or litter to kill intermediate hosts may be beneficial (50). Extreme care should be taken to ensure that feed and water are not contaminated. Removal or effective management of litter can reduce infections. After the old litter has been removed from commercial houses or banked for heating, spraying the exposed soil with a saturated salt solution or acceptable (environmentally compatible) insecticide before replacement of new or piled litter may help control populations of potential intermediate hosts, as well as reduce the numbers of viable nematode eggs. However, there is little documentation of the value of such treatments.

Raising different species or different ages of birds together or in close proximity creates more opportunity for some parasites to prosper. For instance, turkeys should not be raised with any birds likely to harbor patent *Heterakis* infections, due to the turkey's high susceptibility to blackhead disease.

Chemotherapy

Because of the high cost of securing data necessary for FDA approval of new compounds, investigation into the efficacy of anthelmintics has been limited to parasites of incidence and magnitude that would suggest a large potential drug market. Current information on drug efficacy for poultry is therefore limited to treatment of ascarids, capillaria, and heterakids, the most important nematode parasites in commercially grown poultry.

Only a few compounds have been approved by the FDA for use in poultry. Producers should be aware that the use of unapproved drugs is not legal in birds that are intended to produce eggs or meat for market. Recent regulatory changes concerning the extra-label use of drugs have been made in the United States which make it easier for products approved for other animals to be administered under the direction of veterinarians. However, it is necessary to document the status of the parasitism and the lack of available, efficacious, and label-indicated product for control. Extreme care should be taken to obtain current information from authoritative sources before any medication is used. Label directions, dosages, and withdrawal periods must be followed.

Ascaridia galli *and* A. dissimilis

Approved Compounds

Piperazine compounds have been widely used for the treatment of turkey and chicken ascaridiasis for a number of years. In a series of studies conducted at the University of Arkansas, the efficacies of various piperazine salts at dose levels that ranged from 50 to 400 mg/kg bw were tested, and very little efficacy was observed in either chickens or turkeys (35).

Fenbendazole has recently been approved for the treatment of growing turkeys infected with *Ascaridia dissimilis.* The product is given in the feed at 16 ppm, thereby providing an approximate dose rate of 5 to 10 mg/kg bw over 6 days of treatment.

In naturally-infected commercial turkeys, fenbendazole was found to be >98% effective in the removal of adult and larval turkey ascarids (86).

Experimental Data

Fenbendazole administered in the feed at the level of 30 ppm for 4 days or 60 ppm for 3 days was 100% effective against *A. galli* in chickens (58, 85). Drenching chickens with 10% fenbendazole suspension for a dosage of 5 mg/kg bw is effective in the elimination of all parasitic stages of *A. galli*. In a recent study, albendazole drench given to chickens at the rate of 5 mg/kg bw was 100% adulticidal and 88% larvacidal for *A. galli* (Yazwinski, Tucker and Cummins, unpublished data). With prescription, commercial turkeys and chickens are commonly treated with oxfendazole at dose rates of 3.5 mg/kg bw or slightly higher for ascarid control.

Ascaridia galli infections were effectively removed with dl-tetramisole at the dose rate of 40 mg/kg of body weight (8,57). L-tetramisole (levamisole), the more efficacious isomer of tetramisole, was effective against *A. dissimilis* in turkeys when given at the rate of 30 mg/kg of body weight (40). Levels of 0.06% or 0.03% levamisole in the drinking water removed 99% of adult *A. dissimilis,* and 94–98% of larval *A. dissimilis*(57). Twenty-five milligrams of levamisole per kilogram of body-weight was found to be an effective dose rate for the treatment of *A. galli* infections in chickens (10). Levamisole hydrochloride is commonly given under prescription to chickens and turkeys for the treatment of ascarid infections, but at dose rates rarely in excess of 12 mg / kg bw. Controlled study evaluations of levamisole at this dose level have not been reported.

Pyrantel pamoate was not efficacious in the treatment *A. galli* in chickens (75). Conversely, pyrantel tartrate at a single dose of 15–25 mg/kg body weight gave 99.6–100% removal of adult *A. galli* from chicks but was relatively ineffective against the larval stages (56).

Capillaria obsignata *(Threadworms)*

Approved Products

No approved products are currently available for the efficacious removal of *C. obsignata* infections from chickens. Off-label use of other products on a prescription basis is therefore indicated.

Experimental Data

Fenbendazole was reportly more than 97% effective in removing experimental infections of *C. obsignata* when administered to turkeys at 45 ppm for 6 days (52). The compound was greater than 99% efficacious in chickens (58). Other research indicated slightly lower efficacy when lighter breed chickens were fed fenbendazole at 80 ppm for 3 days or 48 ppm for 5 days (85).

Treatment of naturally-infected breeder hens with albendazole in a drench formulation at the rates of 5, 10 or 20 mg/kg bw reduced burdens of *C. obsignata* 90, 91 or 95%, respectively (Unpublished data).

Methyridine injected subcutaneously beneath the wing as a 5% aqueous solution was effective for the removal of *C. obsignata* from pigeons (82). Injections of 25–45 mg methyridine per bird were 99–100% effective against *C. obsignata* in naturally-infected birds, but doses of 23 mg per bird was only 62% effective. The anthelmintic action of methyridine was relatively rapid, as indicated by elimination of the majority of the worms within 24 hours of treatment. Piperazine citrate, phenothiazine, thiabendazole, and bephenium were inactive against *C. obsignata.*

C. obsignata was effectively removed with dl-tetramisole at a dose rate of 40 mg/kg of body weight (8,57). L-tetramisole (levamisole) given to turkeys at the rate of 30 mg/kg of body weight was effective against naturally-acquired infections of *C. obsignata* (40). Levamisole in the drinking water at the levels of 0.06% or 0.03% removed 99–100% of the *C. obsignata* burdens of chickens (57). Twenty-five milligrams of levamisole per kilogram of body weight was found to be effective for the treatment of *C. obsignata* infections in chickens (10).

Heterakis gallinarum *(Cecal worms)*

Approved Products

No approved products are currently available for the efficacious removal of *Heterakis gallinarum* infections from chickens. Off-label use of other products on a prescription basis is therefore indicated.

Experimental Data

Fenbendazole had 100% efficacy in turkeys experimentally infected with *H. gallinarum* and given in the ration at 120 ppm for 3 days or 45 ppm for 6 days (52). The compound had the same efficacy at 30 ppm for 6 days or 60 ppm for 3 days in experimentally infected chickens (58). A separate study indicated 100% efficacy could be achieved when experimentally infected birds were given feed with 30 ppm fenbendazole for 5 days (85).

In a recent study, albendazole, delivered as a drench at the rates of 5–20 mg/kg bw, removed *Heterakis gallinarun* from naturally-infected breeder hens by greater than 90% (Unpublished data).

Heterakis gallinarum was effectively removed with dl-tetramisole at the rate of 40 mg/kg of body weight (8,57). L-tetramisole (levamisole) given to turkeys naturally infected with *H. gallinarum* at the rate of 30 mg/kg of body weight was also effective (40). Levels of 0.06% or 0.03% levamisole in the drinking water removed 99–100% of the *H. gallinarum* (57).

Syngamus trachea *(Gapeworms)*

Mash containing 0.5% thiabendazole fed to 4-week-old turkey poults for 9–20 days removed 98% of the gapeworms from 117 birds (79). The drug appeared effective, whether treatment was initiated on post-infection day 30 or started on the day of infection. Continuous medication of pen-reared birds at dietary levels of 0.1–4% has been recommended but is not economically practical.

Several other compounds have been shown effective against *Syngamus.* Mebendazole was 100% efficacious when fed prophylactically at 0.0064% and curatively at 0.0125% to turkey poults (70). A dietary level of 0.044% for 14 days has also been shown effective.

Cambendazole was found to be more efficacious than thiabendazole or disophenol (21). The level of control with three treatments of cambendazole on days 3–4, 6–7, and 16–17 post-infection was 94.9% in chickens (2 × 50 mg/kg) and 99.1% in turkeys (2 × 20 mg/kg).

Levamisole at a dietary level of 0.04% for 2 days or in the drinking water with a concentration of 2 g/gal for 1 day each month has proven effective in game birds. Fenbendazole at 20 mg/kg for 3 to 4 days is also effective (68).

Other Nematodes

Cambendazole (60 mg/kg) was effective against both adult and larval *Amidostomum anseris* (22). Pyrantel (100 mg/kg) was effective against adults. Some success was also obtained with citarin (40 mg/kg). Mebendazole at 10 mg/kg given for 3 consecutive days completely eliminated *A. anseris* (24). Other benzimidazoles may also be effective against these worms.

Haloxon was 46–100% effective against *C. contorta* in quail

when administered at levels of 0.05–0.5% in the feed for 5–7 days (11). Results were best at the 0.075–0.5% levels. However, the highest concentration was toxic, and one-fourth of the birds died. Single oral doses of the drug were not uniformly effective and produced undesirable side effects, primarily ataxia.

Tetramisole is not effective against *Dispharynx nasuta*, although mebendazole has some efficacy.

Subulura brumpti can be partially controlled with several different tin compounds or tetramisole.

Tetramisole also has some effect against *Strongyloides avium.*

Piperazine has been used against *Tetrameres fissispina.*

Trichostrongylus tenuis can be controlled by cambendazole (30 mg/kg), pyrantel tartrate (50 mg/kg), thiabendazole (75 mg/kg), and citarin (40 mg/kg) (23). Mebendazole was completely effective at 10 mg/kg given for 3 consecutive days (24).

C. Acanthocephalans

The Acanthocephala (thorny-headed worms) live as adults in the intestinal tract of vertebrates. Superficially, they resemble nematodes and cestodes, but many differences are apparent. At the anterior end, they have a retractible proboscis, which bears a considerable number of recurved hooks arranged in rows. The number, form, and arrangement of the hooks are valuable diagnostic characteristics. The body is usually unarmed but may bear small spines on some portion of the external surface. Like tapeworms, this group of worms has no digestive tract. Nutrition is obtained by absorption through the body wall. The sexes are separate. The male is smaller and more slender than the female and often distinguished externally by a bell-shaped bursa that surrounds the genital pore.

All known species of Acanthocephala require one or more intermediate hosts before becoming infective for the final host. Various arthropods, snakes, lizards, and amphibians serve as hosts of the larval stages (66).

Only four species of thorny-headed worms have been reported as parasites of domestic poultry in North America. Three of these were immature forms and may have been the result of accidental (dead end) infections.

Oncicola canis *Kaupp 1909*

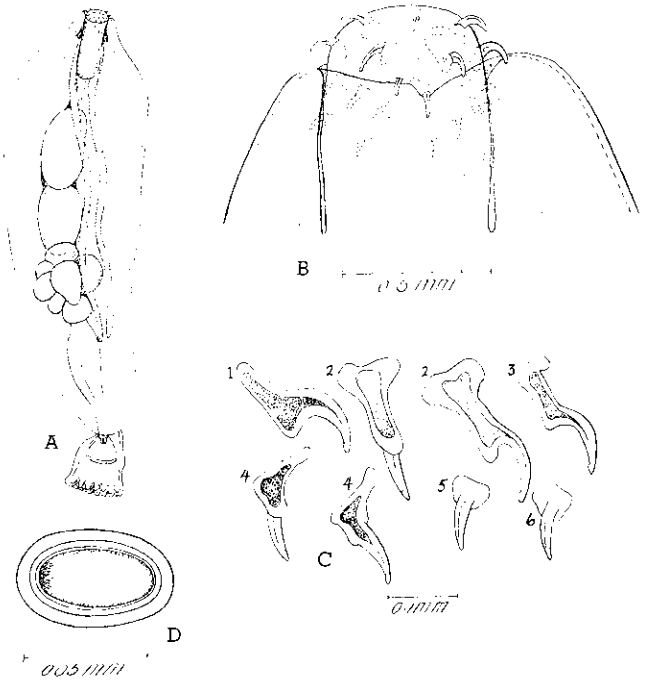
O. canis was found in young turkeys around San Angelo, Texas (Fig. 27.30). The worms were encysted under the epithelial lining of the esophagus in numbers varying from a few to 100 or more. They were reported as the possible cause of death.

Adults normally occur in the dog and coyote. The presence of larval forms in young turkeys suggests an accidental occurrence, with young worms encysting when taken into an unsuitable host.

Larvae of *O. oncicola,* a parasite of South American jungle cats, have been recovered from chickens in Costa Rica.

Prosthorhynchus formosus *(Van Cleave 1918) Travassos 1926*

An immature male and 2 female specimens of *P. formosus* were reported from the small intestine of a chicken necropsied at Vineland, New Jersey. Other bird hosts from which this species has been reported are the flicker (Bowie, Maryland), crow (Washington, D.C.), and robin (New Jersey) (Fig. 27.31). Several authors have suggested that this species is a potential hazard to domestic poultry; however, the level of experimental infections in chickens and turkeys is low (64).



27.30. *Oncicola canis*. A. Male showing reproductive organs. B. Proboscis. C. Hooks from proboscis (numerals indicate row). D. Egg. (Price)

Polymorphus boschadis *(Schrank 1788)*

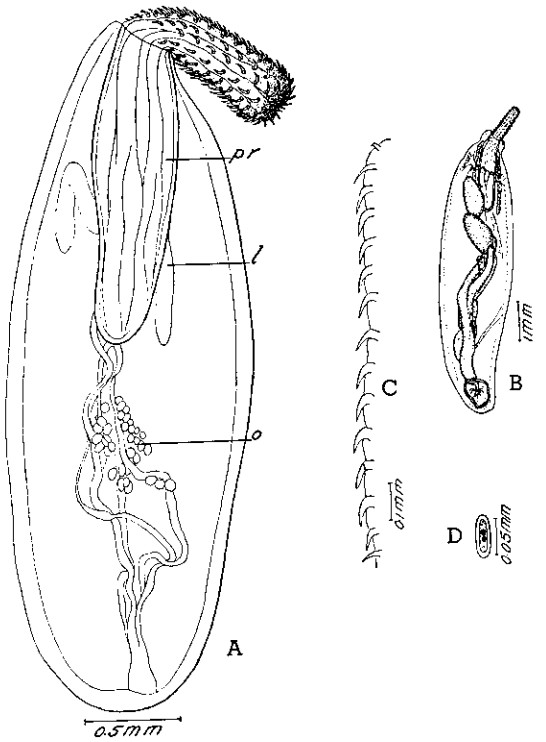
This worm (Fig. 27.32) has been reported from the duck in Canada. It causes serious illness and death in domesticated waterfowl, especially in young birds. It causes an inflammation of the intestine with subsequent anemia and cachexia. Affected birds become visibly sick, with staggering gait and drooping head and wings.

Other Acanthocephalans from Poultry

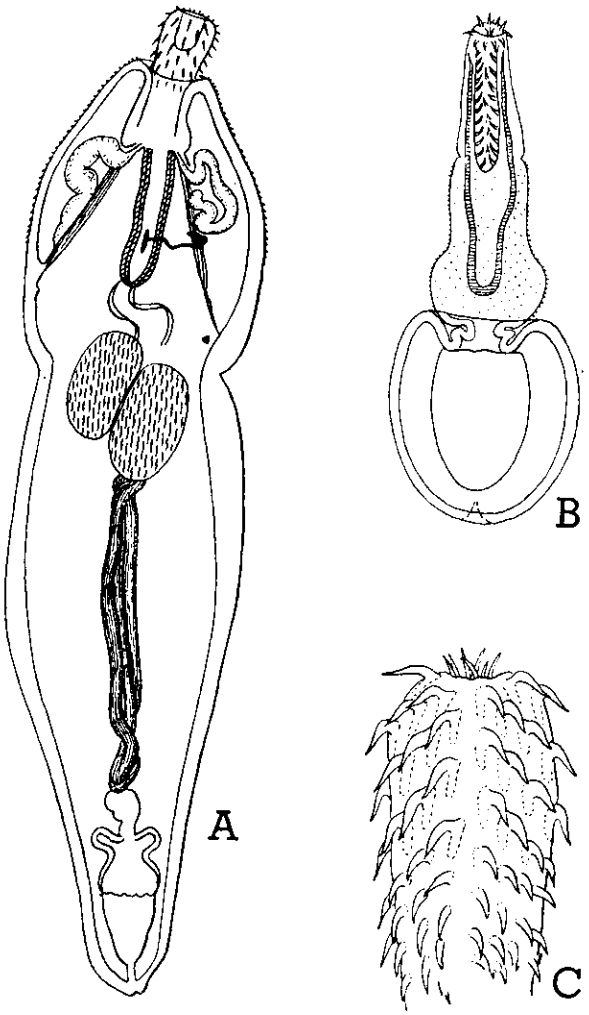
Other acanthocephalans infecting fowl, but not found in North America, include *Leiperacanthus gallinarum, Mediorhynchus gallinarum*, and *Neoschongastia gallinarum* in Asia; *Macracanthorhynchus hirudinaceus* in chickens in Brazil; *Prosthorhynchus rhea* in South America and *Prosthorhynchus transversus* in passerine birds, partridge, and pheasant in Europe. The latter is experimentally infective for chickens.

Public Health Significance

None of the helminths discussed in this section pose a threat to public health. Persons may become accidentally infected with



27.31. *Prosthorhynchus formosus*. A. Young female (l, lemniscus; o, ovary; pr, proboscis receptacle). (Jones) B. Male. C. Hooks from proboscis. D. Egg. (VanCleave)



27.32. *Polymorphus boschadis*. A. Male. B. Larva from *Gamarus pulex.* C. Proboscis of larva. (Luhe)

some of the helminths, especially those that also accidentally infect poultry (e.g. *Baylisascaris* spp.), but fowl will not be the source of the infection as they also represent dead end hosts. Larvae of *Strongyloides avium* may cause a creeping, cutaneous eruption in accidental infections (larva currens), but the lesions soon regress with no lasting pathology. Cutaneous hypersensitivity has been noted with some lab personnel who have been working with this genus (*Strongyloides ransomi*) for long periods of time (51).

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Cestodes and Trematodes

Larry R. McDougald

Introduction

Many species of worm parasites are seen during necropsy examination of the digestive tract or other internal organs of poultry. Some of these are large enough to cause intestinal blockage in severe cases. Others are so small that a hand lens may be required to distinguish them from intestinal contents. If flattened in shape, they are probably "flatworms" belonging to the phylum Platyhelminthes. Tapeworms are in the class Cestoda, and flukes are in the class Trematoda. Accurate identification is essential for effective control. Species identification may give direction to control measures aimed at eliminating the intermediate host, thus breaking the life cycle. Others may require treatment with anthelmintics.

E. E. Wehr authored the chapter on cestodes in earlier editions of this text, and W. W. Price, E. E. Byrd, and Newton Kingston authored a chapter on trematodes. Their contributions to materials included in this edition are gratefully acknowledged.

Cestodes

Most birds are hosts to some species of cestodes or tapeworms (phylum Platyhelminthes/class Cestoda). A high percentage of

Table 27.5. Tapeworms and hosts from poultry in the United States.

Tapeworm	Definitive Hosts (occasional hosts)	Intermediate Hosts	Degree of Pathogenicity
Amoebotaenia cuneata	Chicken (turkey)	Earthworm	Mild
Choanotaenia infundibulum	Chicken (turkey)	Housefly, beetle	Moderate
Davainea proglottina	Chicken	Slug, snail	Severe
Hymenolepis carioca	Chicken (turkey, bobwhite quail)	Stable fly, dung beetle	Unknown
H. cantaniana	Chicken (turkey, peafowl, bobwhite quail)	Beetle	Mild or harmless
Raillietina cesticillus	Chicken (turkey, guinea fowl, bobwhite quail)	Beetle	Mild or harmless
R. tetragona	Chicken (guinea fowl, peafowl, bobwhite quail, turkey)	Ant	Moderate to severe
R. echinobothrida	Chicken (turkey)	Ant	Moderate to severe
R. magninumida	Guinea fowl (chicken, turkey)	Beetle	Unknown
Davainea meleagridis	Turkey	Unknown	Unknown
Drepanidotaenia watsoni	Wild turkey	Unknown	Unknown
Imparmargo baileyi	Wild turkey	Unknown	Unknown
Raillietina georgiensis	Wild turkey (domestic turkey)	Ant	Unknown
R. ransomi	Wild turkey	Unknown	Unknown
R. williamsi	Wild turkey	Unknown	Unknown
Metroliasthes lucida	Turkey (guinea fowl, chicken)	Grasshopper	Unknown
Diorchis nyrocae	Wild and domestic duck	Copepod crustacean	Unknown
Fimbriaria fasciolaris	Duck (chicken)	Copepod crustacean	Unknown
Hymenolepis anatina	Wild and domestic duck	Freshwater crustacean	Severe
H. compressa	Duck, goose	Unknown	Unknown
H. collaris	Wild and domestic duck (chicken)	Freshwater crustacean (snail = auxiliary)	Unknown
H. coronula	Duck	Crustacean, snail	Unknown
H. lanceolata	Goose, duck	Crustacean	Severe
H. megalops	Duck	Unknown	Unknown
H. parvula	Wild and domestic duck	Leech	Unknown

chickens or turkeys may be infected with tapeworms if they are reared on range or in backyard flocks. These parasites are found more frequently in warmer seasons, when intermediate hosts are abundant. Many species of tapeworms are now considered rare in intensive poultry-rearing regions because the birds do not come in contact with intermediate hosts. Beetles and houseflies inhabiting poultry houses still act as intermediate hosts for the 2 large chicken tapeworms known only by the scientific names *Raillietina cesticillus* and *Choanotaenia infundibulum.*

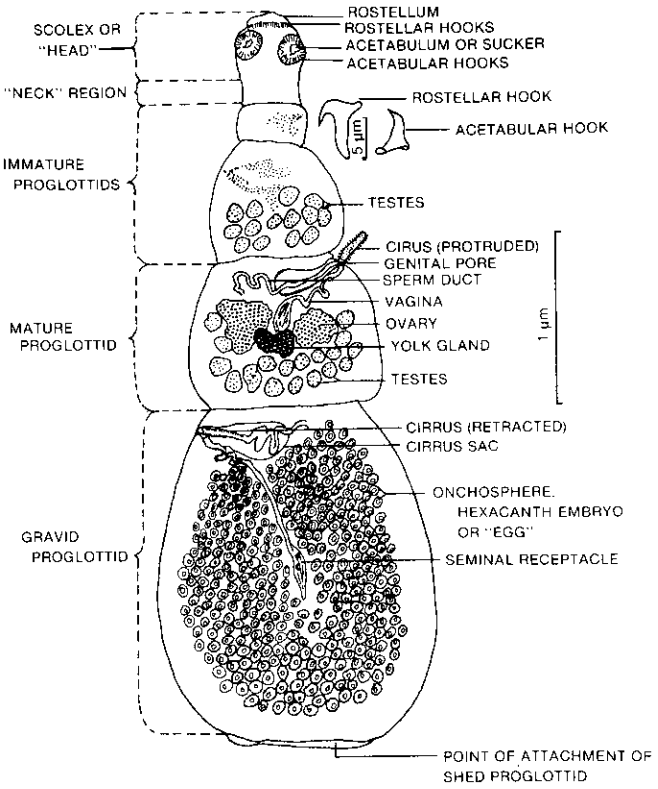
Some infections of the larger tapeworms may appear to block completely the intestine of an infected bird, but mortality from cestodiasis or long-term effects are rare. Different species vary considerably in pathogenicity, so species identification is worthwhile.

Diagnosticians are often satisfied with a diagnosis of "cestodiasis" or "taeniasis" without making further attempts at identification. However, prevention and control strategies may vary with each species of tapeworm. Only after the species has been determined can an assessment of flock damage and possible control measures be considered (Table 27.5). For identification of the less common species, specialized textbooks may be needed to supplement the keys and illustrations included in this text (8, 11, 15, 16).

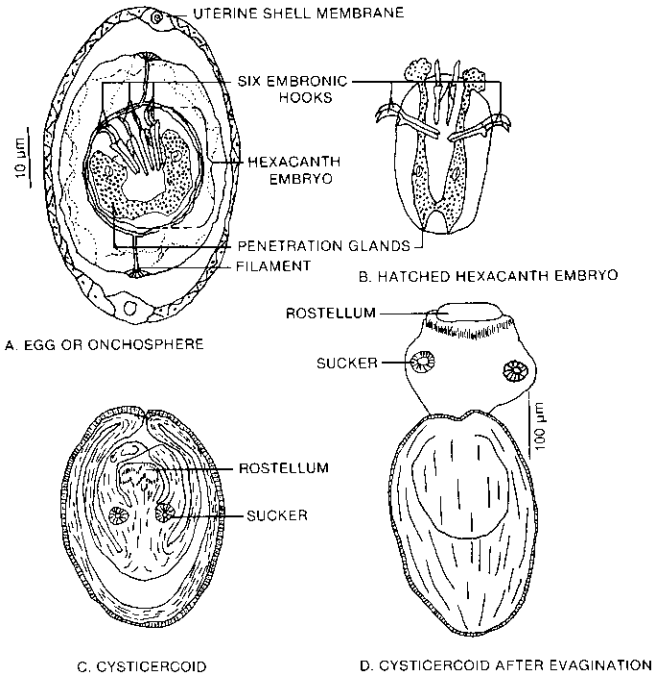
Tapeworms or cestodes are flattened, ribbon shaped, usually segmented worms. The term *proglottid* is used to describe these individual segments, because the latter term is defined otherwise by classic zoologists (see Fig. 27.33). One to several gravid proglottids are shed daily from the posterior end of the worm. Each proglottid contains one or more sets of reproductive organs, which may become crowded with a mass of eggs as the maturing proglottid becomes a gravid proglottid.

Tapeworms are characterized by complete absence of a digestive tract and obtain their nourishment by absorption from the gut contents of the host. Although the duodenum, jejunum, or ileum is the usual site for attachment, 1 species (*Hymenolepis megalops*) from ducks is found in the cloaca or bursa of Fabricius. Birds become infected by eating an intermediate host, thus allowing the larval stage of the tapeworm access to the intestine. This larval tapeworm is known as a cysticercoid (see Fig. 27.34C). The intermediate host may be an insect, crustacean, earthworm, slug, snail, or leech depending upon the species of tapeworm.

Most cestodes are host specific for a single or a few closely related birds. Identification of the parasite to genus and species will help pinpoint the intermediate host. The diagnostician then may be able to suggest practical control measures. Completion of a two-host life cycle depends upon a unique set of ecologic conditions which juxtapose the host and the intermediate host. Thus, minor changes in flock management may cause a break in the life cycle and comprise an effective control measure.



27.33. Adult tapeworm (*Davainea proglottina*). Although readily seen with the naked eye, this species has been called a "microscopic tapeworm," because it is small and often overlooked.



27.34. Larval stages of the chicken tapeworm (*Raillietina cesticillus*). A. The egg is encapsulated by a membrane derived from the uterus wall. Eggs are occasionally found free in feces, but more often enclosed within a gravid proglottid. B. Hexacanth embryos escape from shell membranes; active hooks and enzymes from secretory glands assist in penetration of gut wall of the beetle intermediate host. C. Cysticercoid that has developed in the hemocoele of a beetle. D. Scolex in the cysticercoid has evaginated after exposure to bile and enzymes in gut of the fowl.

History, Incidence, and Distribution

More than 4000 species of tapeworms have been described from animals (14), with many of the earlier species bearing the genus name *Taenia.* Because no poultry tapeworms are currently listed in this genus, the term *taeniasis* is no longer appropriate, and the term *cestodiasis* would be a better substitute for infection with poultry tapeworms. Slender threadlike forms (*Hymenolepis carioca*) may require some magnification to distinguish individual proglottids, thus indicating that they are tapeworms. Some small forms (e.g., *Davainea proglottina*) are almost microscopic.

Classification

More than 1400 species of tapeworms have been described from wild and domestic birds. Because most of them have no common name, they are best recognized by their genus and species names.

Three families (Davainidae, Dilepididae, and Hymenolepidae) and 10 genera (*Amoebotaenia, Choanotaenia, Davainea, Diorchis, Drepanidotaenia, Imparmargo, Metroliasthes, Raillietina, Hymenolepis, Fimbriaria*) are recognized here, as they may appear in birds brought to diagnostic laboratories in the United States.

Morphology and Life Cycles

Adults

The anatomic features needed to identify poultry tapeworms are illustrated by describing *Davainea proglottina* (Fig. 27.33). This species differs from most other tapeworms in possessing only 1 or 2 each of immature, mature, and gravid proglottids compared with dozens or hundreds in other species. The entire connected chain of proglottids is called a strobila. Besides the strobila, 2 other regions, the scolex and the neck, are recognized. Anchorage is accomplished by the scolex with the assistance of 4 pairs of suckers or acetabula, which may possess 1 or 2 rows of acetabular hooks. If hooks are present, the species is described as armed; if absent, it is unarmed. A plunger-shaped organ known as the rostellum is frequently present at the anterior end. The rostellum may assist in anchorage by means of 1 or 2 rows of rostellar hooks and by the suction created by partial withdrawal of the rostellum into the scolex. The neck is an undifferentiated area between the scolex and the strobila from which new proglottids proliferate.

A set of both male and female reproductive organs are found in each proglottid. Morphologic differences in size and location of these organs are used in taxonomic descriptions of different

species. Older gravid proglottids containing numerous eggs are shed individually or in short chains late in the day after the worm has absorbed and stored nutrients from the gut contents of the host. *D. proglottina* generally sheds 1 gravid proglottid per day, and *Raillietina cesticillus* may produce as many as 10–12.

Onchosphere

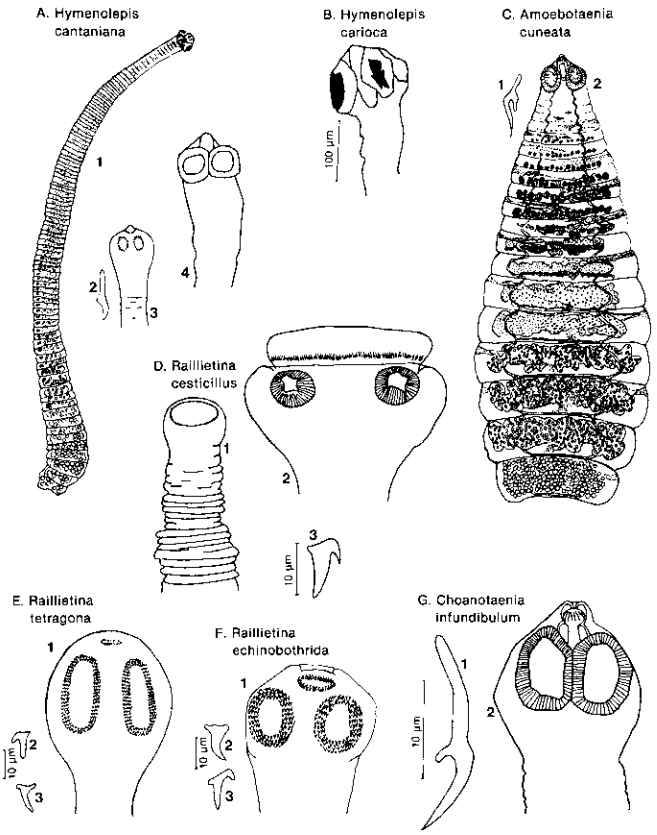
Within the uterus, the fertilized egg develops into a multicellular embryo called an onchosphere or hexacanth embryo. The onchosphere is a multicellular larva containing penetration glands and numerous muscular attachments to activate the hooks. Each gravid proglottid may contain several hundred of these multicellular embryos or eggs. Distinctive membranes (Fig. 27.34A) surrounding the eggs may be useful in identifying the species.

Cysticercoid

Intermediate hosts such as beetles, houseflies, slugs, or snails become infected by swallowing individual eggs from the feces, or they devour the entire proglottid after being attracted by odor or movement. The 6-hooked embryo hatches from the egg in the gut of the intermediate host and penetrates the gut wall. The larva reorganizes and changes in polarity to become a cysticercoid in about 2 weeks (Fig. 27.34C,D). The cysticercoid remains within the body cavity of the intermediate host until the latter is eaten by the bird host. In the digestive tract the cysticercoid is activated by bile and attaches to the intestine to begin the formation of a strobila. The first gravid proglottids appear in the feces 2–3 weeks after the cysticercoid is swallowed.

Diagnosis and Identification

Distinctive characteristics of tapeworms may best be demonstrated by examining 1) the scolex (Figs. 27.33, 27.35), 2) the eggs (Figs. 27.34, 27.37), or 3) individual proglottids of recently shed, and whole live specimens (Figs. 27.33, 27.36) (11). Although differential staining can be used to show the internal organs of mature proglottids, this procedure is too slow for most diagnostic laboratories. Preservation in alcohol or formalin, although required before staining, often obscures useful characteristics needed for rapid identification. The intestine is best opened with scissors under water, thus permitting the strobila to float free, revealing the area to which the scolex is attached. Recovery of the scolex is worth considerable effort, as its characteristics alone may indicate the species. Freeing the scolex may be accomplished by 1) teasing apart the mucosa with 2 dissecting needles, 2) cutting a deep gouge into the mucosa under the attachment point with a sharp scalpel, or 3) leaving the intestine submerged in saline for a few hours in the refrigerator. Wet-mount preparations of the scolex examined under a coverglass with 3100 or higher magnification may reveal sufficient characteristics to make a species identification. Hook characteristics may require measurement with an ocular micrometer under higher magnification. Semipermanent cleared preparations of scolices may be made by using a drop of Hoyer's solution (prepared by adding to 50 mL of distilled water the following ingredients in this order: 30 g gum arabic flakes; 200 g chloral hydrate; and 20 g glycerin).

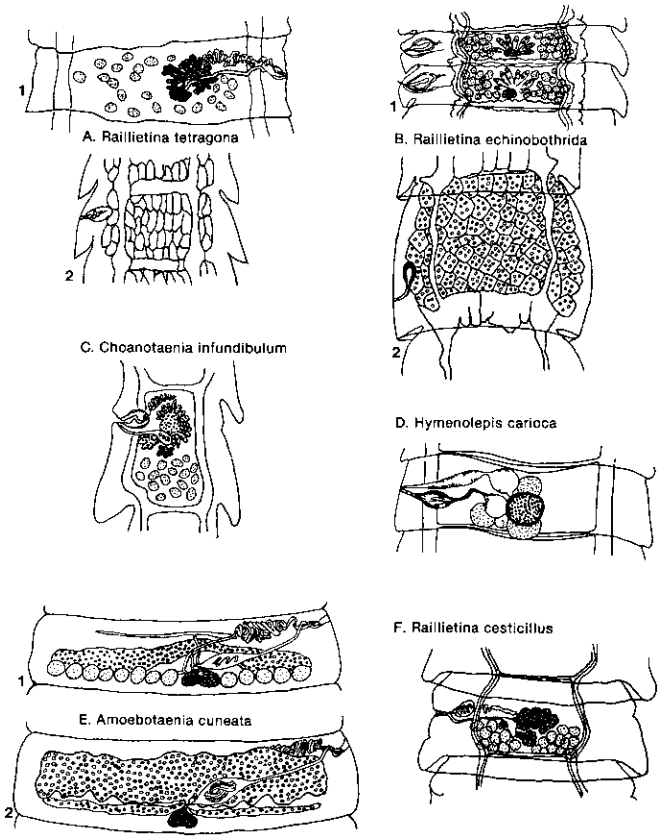


27.35. Tapeworms of chickens. Scolex characteristics: A. *Hymenolepis cantaniana*. 1. Scolex and strobilia (Ransom); 2. Hook (Yamaguti); 3. Scolex (Neveu-Lemaire); 4. Scolex (Wehr). B. *H. carioca* scolex. C. *Amoebotaenia cuneata* (Monnig). 1. Rostellar hook; 2. Entire worm. D. *Raillietina cesticillus*. 1. Scolex (Ackert); 2. Scolex (Monnig); 3. Rostellar hook (Ransom). E. *R. tetragona*. 1. Scolex (Monnig); 2.B3. Rostellar and acetabular hooks (Ransom). F. *R. echinobothrida*. 1. Scolex (Monnig); 2.B3. Rostellar and acetabular hooks (Ransom). G. *Choanotaenia infundibulum*. 1. Hook (Ransom); 2. Scolex (Monnig).

Distinctive egg characteristics may be demonstrated by teasing apart a gravid proglottid under a coverglass (Fig. 27.37). Wet preparations of mature or gravid proglottids under low magnification may reveal diagnostic characteristics such as the location, size, and shape of the cirrus pouch and the location of the genital pore and the gonads. If further details of the internal structure of the proglottid are required for identification, it may be necessary to kill, fix, stain, destain, dehydrate, and permanently mount the specimen (1).

Tapeworms of Chickens

A dichotomous key is given to the eight species of tapeworms commonly found in chickens from the continental United States. In such keys, successive selections must be made between 1a and 1b, 2a and 2b, etc., until a species name is designated. After



27.36. Mature and gravid proglottids of chicken tapeworms. A. *Raillietina tetragona*. 1. Mature proglottid (Ransom); 2. Gravid proglottid showing egg capsules (Neveu-Lemaire). B. *R. echinobothrida*. 1. Mature proglottid (Fuhrmann); 2. Gravid proglottid (Lang). C. *Choanotaenia infundibulum* (Fuhrmann). D. *Hymenolepis carioca* (Sawada). E. *Amoebotaenia cuneata*. 1. Mature proglottid; 2.Gravid proglottid filled with eggs (Fuhrmann). F. *Raillietina cesticillus*: mature proglottid (Monnig).

viewing a portion of the worm under the microscope, make a comparison of the appropriate figures organized under scolices (Fig. 27.35), eggs (Fig. 27.37), or proglottids (Fig. 27.36). With rare species, additional descriptions from other texts may be required (16).

Key to Species

1a. Minute forms, less than 1 cm long. A very limited number of proglottids with the terminal proglottid being gravid with eggs 2

1b. Longer than 1 cm 3

2a. Wedge-shaped worm. Contains about 20 proglottids. Posterior proglottids wide, short *Amoebotaenia* (Figs. 27.35C,27.36E, 27.37) . . . . . *.cuneata*

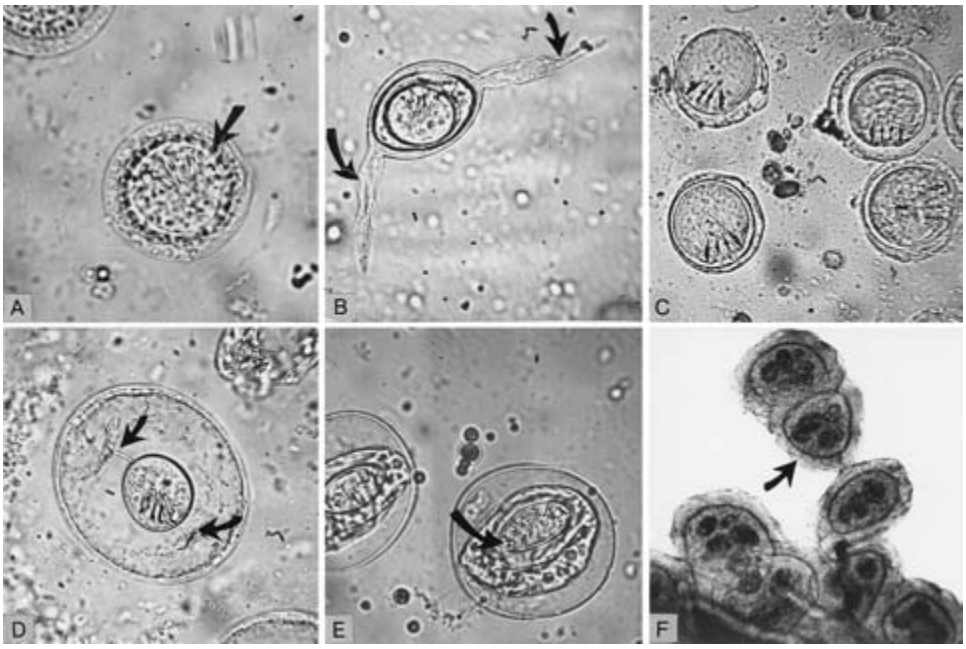
2b. Contains only 2–5 proglottids, rarely 9. Posterior proglottids as *Davainea* long as wide (Fig. 27.33) . . . . . *.proglottina*

3a. Threadlike, never more than 1.5 mm wide; fragile scolex is usually lost; often more than 100 worms in a single bird; proglottids short and wide, genus *Hymenolepis* 4

3b. Robust worms, gravid proglottids wider than 2 mm 5

4a. Mature worms with gravid proglottids present, less than 12 mm long (Fig. 27.35A) . . . . . *.H. cantaniana*

4b. Mature specimens with a total length including gravid proglottids of more than 12 mm (Figs. 27.35B, 27.36D) . . . . . *.H. carioca*



27.37. Eggs of chicken tapeworms (high power). A. *Amoebotaenia sphenoides* showing distinctive granular layer. B. *Choanotaenia infundibulum* with elongated filaments. C. *Davainea proglottina*. D. *Raillietina cesticillus*: Gravid proglottid showing distinctive funnel-shaped structures between membranes. E. *Hymenolepis carioca* or *H. cantaniana* showing football-shaped embryophore with granular accumulations at the poles. F. Capsules containing 6–12 eggs. Found in the chicken (*Raillietina tetragona*, *R. echinobothrida*) and 2 turkey tapeworms (*R. georgiensis*, *R. williamsi*).

5a. 5–12 embryos enclosed in single capsule; verify by opening terminal proglottid; view under a coverglass (Fig. 27.37F) 6
5b. Embryos in single egg capsules enclosed in distinct membranes (Examine under high power) 7
6a. Cirrus sac small (75–100 mm long). Suckers markedly oval in shape (Figs. 27.35E, 27.36A) . . . . . .*R. tetragona*
6b. Cirrus sac large (130–180 mm). Suckers round (Figs. 27.35F, 27.36B) . . .*R. echinobothrida*
7a. Outer membrane prolonged in 2 elongated filaments (Fig. 27.37B) . . . . . *Choanotaenia .infundibulum*
7b. Outer membrane smooth and round, 2 elongated filaments Fig. 27.34A, 27.37D) . . . . . .*R. cesticillus*

Species descriptions are given for these 8 chicken tapeworms to assist in verifying tentative identifications.

Amoebotaenia cuneata *(Linstow 1872)*

Diagnostic Characteristics

This short (less than 4 mm, 25–30 proglottids) tapeworm may be recognized as whitish projections among the villi of the duodenum (Fig. 27.35C); a triangular anterior end with a pointed scolex gives the entire worm a wedge-shaped anterior. Suckers unarmed, rostellum armed with a single row of 12–14 distinctive hooks 25–32 mm long, 12–15 testes located transversely in a single row across the posterior end of the proglottid (27.36E), genital pores usually alternate regularly, located at extreme anterior point of proglottid margin; 6-hooked single embryos, surrounded by a distinctive granular layer (Fig. 27.37A); embryonal hooks, 6 mm.

Life History

Several species of earthworms belonging to the genera *Allotophora, Pheritima, Ocnerodrilus,* and *Lumbricus* act as intermediate hosts for this tapeworm. Literature descriptions of pathogenicity range from "comparatively slight" to "cause of death." No controlled experiments have been reported.

Choanotaenia infundibulum *(Bloch 1779)*

Diagnostic Characteristics

This large robust tapeworm is extremely white and is readily seen attached to the upper half of the intestine: Mature worms up to 23 cm long; large rostellum armed with a single row of 16–22 large (25–30 mm) hooks, suckers unarmed (Fig. 27.35G); genital pores irregularly alternate; 25–60 testes are grouped in posterior portion of proglottid (Fig. 27.36C); eggs are with distinctive elongated filaments (Fig. 27.37B); and embryonal hooks are 18 mm long.

Life History and Pathogenicity

Houseflies and several species of beetles are proven natural hosts. Other insects including 9 families of beetles, grasshoppers, and termites are proven experimental hosts. Gravid proglottids are released 13 days after swallowing an infected fly. No controlled experiments testing pathogenicity have been reported.

Davainea proglottina *(Davaine 1860)*

Diagnostic Characteristics

This microscopic tapeworm may be recognized in the duodenal mucosa by protrusion of the gravid proglottids above the villi if the open intestine is floated in water. Eggs are without distinctive membranes, but embryonal hooks are distinctive, 10–11 mm long (Fig. 27.37C). Mature worms measure up to 4 mm long; never with more than 9 proglottids; suckers are armed with 3–6 rows of hooks (Fig. 27.33); the rostellum is armed; genital pores regularly alternate and are located near the anterior margin; and the cirrus is disproportionately large.

Life History

Several species of slugs and snails host larval stages of this tapeworm. More than 1500 cysticercoids have developed along the digestive tract of susceptible slugs, where they have remained infective for more than 11 months. Tapeworms may live as long as 3 years; more than 3000 worms have been recovered from a single bird.

Pathogenicity

This parasite is one of the more harmful species in young birds. In controlled experiments, a 12% reduction in growth rate has been reported (5). Uncontrolled reports include emaciation, dull plumage, slow movements, breathing difficulties, thickened mucosal membranes that produce hemorrhage and fetid mucus, leg weakness, paralysis, and death.

Hymenolepis cantaniana *(Polonio 1860)*

Diagnostic Characteristics

This short hymenolepid tapeworm (maximum length 2 cm) superficially resembles the longer *H. carioca.* It is usually listed as unarmed, but rostellar hooks have been described by European investigators (Fig. 27.35A); the fragile rostellum is frequently lost; genital pores are unilateral, anterior to middle of proglottid; eggs are similar to those of *H. carioca*; embryonal hooks measure 13–14 mm.

Life History

Dung beetles (Scarabeidae) are intermediate hosts; each beetle may carry 100 or more cysticercoids. A unique larval development involves budding, which produces many cysticercoids from a single onchosphere. This tapeworm is considered relatively nonpathogenic, although no controlled experiments have been reported.

Hymenolepis carioca *(Magalhaes 1898)*

Diagnostic Characteristics

Several thousand specimens of this extremely slender species have been found in the duodenum of a single chicken or turkey. The worm is so slender (about 1 mm in diameter) that the hundreds of inconspicuous proglottids look more like a thread than a worm. Suckers are unarmed; rostellar sacs are present; rostellum

is rudimentary (Fig. 27.35B); there are three testes, usually in a straight row; genital pores are unilateral, located anterior to middle of proglottid margin (Fig. 27.36D); an inner membrane enveloping the onchosphere is elongated into a football shape with granular deposits at poles (Fig. 27.37E); embryonal hooks measure 10–12 mm.

Life History
Twenty-six species belonging to nine families of beetles and one species of termite are experimental or natural intermediate hosts; dung and ground beetles are the most common source of infection. Reports incriminating the housefly are probably erroneous.

Pathogenicity
Experimental infections establishing several hundred worms per bird had no effect on weight gains. These results indicate that this species is relatively nonpathogenic.

Raillietina cesticillus *(Molin 1858)*

Diagnostic Characteristics
Scolex of this large robust tapeworm (up to 15 cm long) embeds deeply in the mucosa of the duodenum or jejunum. The distinctive, wide, flat, rostellum bears a double row of 300–500 hammer-shaped hooks. The flattened rostellum acts as a retractable piston drawing into an outer sleeve of the scolex, thus, providing a firm grip on the mucosa (Fig. 27.35D1,D2); there are 4 unarmed weak suckers; genital pores alternate irregularly (Fig. 27.36F); there are 20–30 testes posteriad in proglottid; single eggs are encapsulated in uterine membranes; and mature eggs have 2 distinctive funnel-shaped filaments between the middle and inner membranes (Fig. 27.37D).

Life History
More than 100 species of beetles belonging to 10 families are proven natural or experimental intermediate hosts. A minute histerid beetle (*Carcinops pumilio*) is the natural intermediate host in broiler houses. The darkling beetle (*Alphitobius diaperinus*), grasshoppers, ants, and lepidopterous larvae have proved negative as experimental hosts. As many as 930 cysticercoids have been found in a single ground beetle.

Pathogenicity
Early reports attribute this parasite with causing emaciation, degeneration, and inflammation of villi, reduction of blood sugar and hemoglobin, and reduced growth rate. None of these early reports could be confirmed in extensive controlled experiments with broilers and layers maintained on optimum nutritional diets (2). Experimental infections (135 worms/bird) produced by feeding 300 cysticercoids caused no reduction in weight gain in broilers or reduced egg production in layers when compared with uninfected controls.

Raillietina tetragona *(Molin 1858)*

Diagnostic Characteristics
These are moderately large tapeworms measuring up to 25 cm long × 3 mm wide. Scolex (Fig. 27.35E1) anchors in the posterior half of the intestine; the rostellum is armed with 90–100 hooks, 6–8 mm long, arranged in a single or double row (Fig. 27.35E2); suckers are oval shaped, armed with 8–12 rows of minute hooks, 3–8 mm long (Fig. 27.35E); genital pores are usually unilateral (Fig. 27.36A); the uterus breaks up into capsules containing 6–12 eggs (Figs. 27.36A2, 27.37F), similar to *R. echinobothrida* from chickens and *R. williamsi* and *R. georgiensis* from turkeys; and the cirrus sac is small (75–100 mm long), more anterior in proglottid margin than with *R. echinobothrida.*

Life History
Several species of small ants that nest under rocks or boards act as intermediate hosts. The minimum prepatent period after feeding cysticercoids to chickens is 13 days.

Pathogenicity
Weight loss was demonstrated in controlled experiments (9) with white leghorns and hybrids infected with an average of 12–16 worms/bird. Decreases in egg production in 4 breeds of hens occurred after administering 50 cysticercoids/bird, causing reduced glycogen levels in livers and the intestinal mucosa of infected chickens.

Raillietina echinobothrida *(Megnin 1881)*

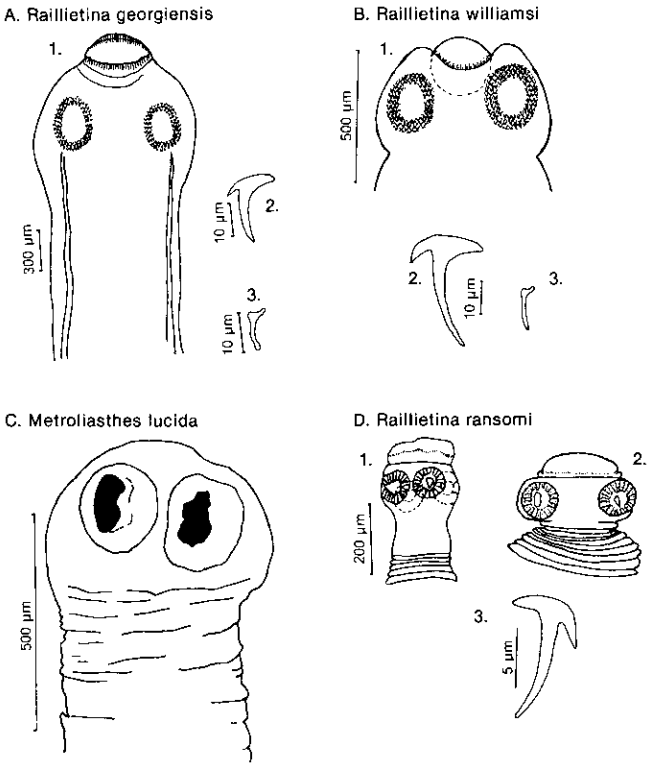
Diagnostic Characteristics
This species resembles *R. tetragona* but differs in the following characteristics: The strobila is larger (34 cm long × 4 mm wide); the scolex has rounded suckers containing 200–250 hooks, 10–13 mm long (Fig. 27.35F) with 8–15 rows of hooks 5–15 mm long (Fig. 27.35F2,3); genital pores are in the posterior half of the proglottid (Fig. 27.36B2); the cirrus sac is large (130–180 mm long); and gravid proglottids frequently loosen from each other in the center, making a windowlike arrangement not found in *R. tetragona.*

Life History
As with *R. tetragona,* numerous species of ants have been found naturally infected with cysticercoids. Concurrent infections with both *R. echinobothrida* and *R. tetragona* cysticercoids have been found in ants.

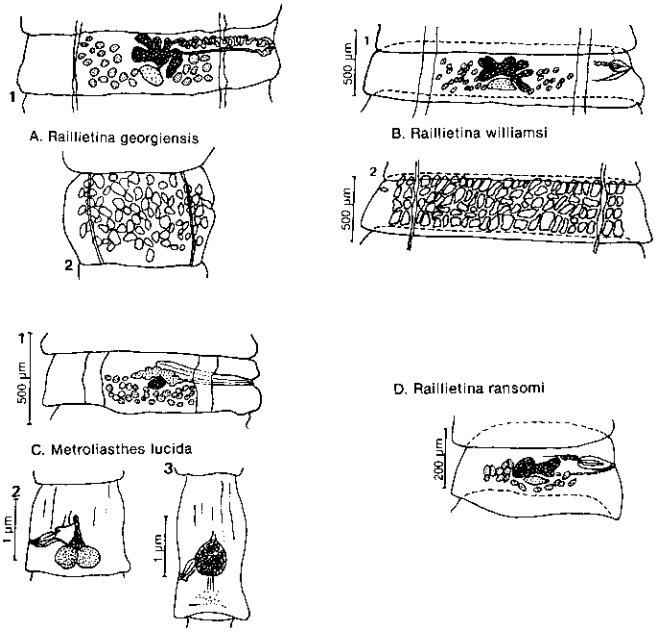
Pathogenicity
R. echinobothrida is usually listed as one of the most pathogenic tapeworms, because its presence has often been associated with nodular disease of chickens. Nadakal *et al.* (10) reported parasitic granulomas approximately 1–6 mm in diameter at the sites of worm attachment 6 months after experimental infection with 200 cysticercoids. The condition was associated with catarrhal hyperplastic enteritis as well as lymphocytic, polymorphonuclear, and eosinophilic infiltration.

Tapeworms of Turkeys

Six species of tapeworms from domestic and/or wild turkeys have been reported from the United States (12). Because these tapeworms are readily transferred between wild and domestic



27.38. Scolices of turkey tapeworms. A. *Raillietina georgiensis*. 1. Scolex; 2. Rostellar hook; 3. Acetabular hook (Reid and Nugara). B. *R. williamsi*. 1. Scolex; 2. Rostellar hook; 3. Acetabular hook (Williams). C. *Metroliasthes lucida* scolex (Ransom). D. *R. ransomi*. 1.–2. Scolex; 3. Rostellar hook (Williams).



27.39. Mature and gravid proglottids of domestic and wild turkey tapeworms. A. *Raillietina georgiensis* (Reid and Nugara). B. *R. williamsi*. 1. Mature proglottid; 2. Gravid proglottid showing position of egg capsules, each containing several eggs (Williams). C. *Metroliasthes lucida*. 1. Mature proglottid; 2. Proglottid showing two-part uterus and developing parauterine organ; 3. Gravid proglottid (Ransom). D. *R. ransomi* mature proglottid (Williams).

turkeys, wild turkeys provide a reservoir for these parasites of domestic birds. No controlled experiments on pathogenicity have been reported for any species. Descriptions included here are limited to the 2 species with known life cycles. Scolex (Fig. 27.38) and proglottid characteristics (Fig. 27.39) of different species are organized in separate figures to facilitate comparisons if complete specimens are unavailable.

Raillietina georgiensis *(Reid and Nugara 1961)*

Description and Diagnostic Characteristics

This species is a large (15–38 cm long × 3.5 mm wide) robust tapeworm from domestic and wild turkeys. Scolex (Fig. 27.38A) is armed with a double row of 230 moderate length (12–23 mm) rostellar hooks and 8–10 circles of acetabular hooks, 8–13 mm long (Fig. 27.38A2,A3); genital pores are unilateral, located in middle of the proglottid (Fig. 27.39A); eggs are in uterine capsules, similar to *R. tetragona* and *R. echinobothrida.*

Life History

A small brownish ant (*Pheidole vinelandica*) that frequents turkey ranges has been found naturally infected; gravid proglottids appear in droppings within 3 weeks after turkeys have fed on infected ants. This tapeworm was introduced to a domestic farm by wild turkeys.

Pathogenicity

Enteritis is present if parasites are found in large numbers. Some host damage is assumed on the basis of a close relationship to *R. echinobothrida* from chickens.

Metroliasthes lucida *(Ransom 1900)*

Description and Diagnostic Characteristics

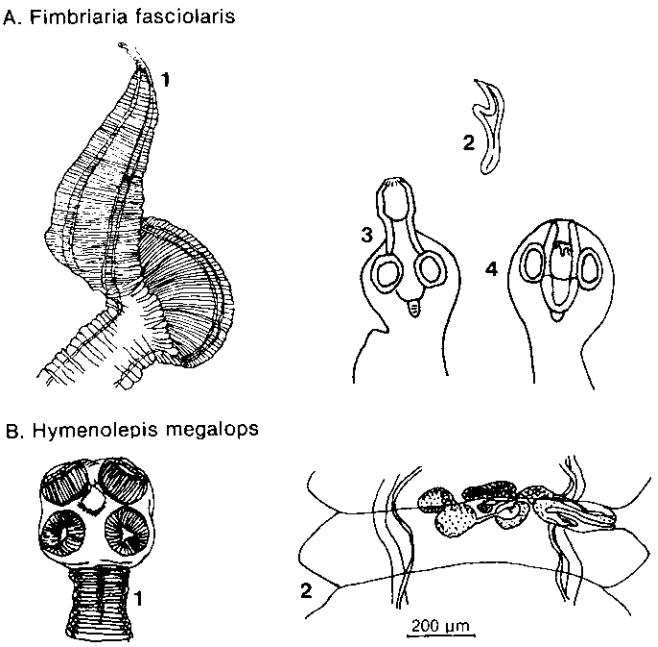
This species is a long tapeworm (20 cm) from turkeys and guinea fowl, rarely in chickens. There are unarmed scolex and suckers, 200–250 mm in diameter (Fig. 27.38C); genital pores irregularly alternate, near middle of margin in mature proglottids but posterior in gravid proglottids; uterus consists of 2 sacs side by side, visible to the naked eye in gravid proglottids, and is known as the parauterine organ (Fig. 27.39C2,C3); eggs have three membranes, 75 × 50 mm.

Life History

Several species of grasshoppers serve as intermediate hosts; cysticercoid development requires 15–42 days depending on temperature. Pathogenicity is unknown.

Tapeworms of Ducks and Geese

Domestic ducks and geese frequently become infected with numerous species of tapeworms introduced by wild ducks and



27.40. Tapeworms of ducks and geese. A. *Fimbriaria fasciolaris*. 1. Pseudoscolex showing irregular distension of the anterior end and the minute scolex (Todd); 2. Rostellar hook (Fuhrmann); 3. Scolex with rostellum extended; 4. Scolex with rostellum withdrawn (Neveu-Lemaire). B. *H. megalops*. 1. Scolex; 2. Mature proglottid (Yamaguti).

geese. Some of these species have occasionally been reported in chickens. Two of the more common species are described in this section. Life cycles usually involve crustaceans or other aquatic invertebrates. No controlled pathogenicity studies have been made on any of these species.

Fimbriaria fasciolaris (Pallas 1781)

Description and Diagnostic Characteristics

This large (5–43 cm long × 1–5 mm wide) twisted tapeworm of ducks also occurs in chickens and 31 species of wild birds. This distinctive flaring anterior neck region is known as the pseudoscolex; strobila is unsegmented, but cross-striations give the impression of segmentation (Fig. 27.40A1); there are minute scolex (Fig. 27.40A3,A4) attached to pseudoscolex, 100–130 mm wide; suckers are unarmed; the retractile rostellum has 10–12 hooks 17–22 mm long (Fig. 27.40A2); genital pores are unilateral and closely crowded together; onchospheres are 35–45 mm in diameter; hooks are 16 mm long.

Life History

Cysticercoids develop in copepod crustaceans (*Diaptomus* sp., *Cyclops* sp.); intermediate hosts are ingested with drinking water to infect the definitive host. Pathogenicity is unknown.

Hymenolepis megalops *(Nitzsch, in Creplin 1829)*

Description and Diagnostic Characteristics

This cosmopolitan tapeworm of waterfowl (Fig. 27.40B) is 3–6 mm long and readily recognized by the large scolex (1–2 mm wide) attached to the cloaca or the bursa of Fabricius. Suckers and rostellum are unarmed, the latter containing a rudimentary central pit; eggs are not in capsules.

Life History

Onchospheres develop into cysticercoids after 18 days in ostracod crustacea. The definitive host is infected by eating ostracods.

Pathogenicity

Reports range from "severe damage" to "mortality if other cestodes (*H. coronula, H. furcigera*) are also present."

Prevention and Control

The change in production methods in commercial poultry, from backyard or range management to confinement rearing in large houses, has brought on marked reductions in tapeworm infections in chickens and turkeys. These birds no longer have easy access to the required insect or other invertebrate hosts for most cestode and trematode parasites. *Davainea proglottina,* one of the most pathogenic species, was reported from 23% of the chickens submitted to the diagnostic laboratory in New York in 1932. No cases have been found in recent years, probably because poultry no longer has easy access to garden slugs. We can expect a more diverse fauna of cestodes in birds reared under free range conditions.

Prevention of contact with the intermediate host is the first step to consider in tapeworm control. Elimination of intermediate hosts may provide additional benefits besides tapeworm control. If *Choanotaenia infundibulum* appears in a cage layer facility, housefly control will benefit the producer by preventing nuisance and public health complaints (see Chapter 32). If *Raillietina cesticillus* tapeworms appear in broiler houses, beetle control measures for the darkling beetle (*Alphitobius diaperinus*) may also reduce populations of the true intermediate host *Carcinops pumilio,* a minute histerid beetle. Identification of worm species will help to suggest control measures, where the control of the intermediate host is to be redommended.

Treatment

In the United States, there are no products for use in feed for treatment of tapeworms in poultry. Historically, butynorate (dibutyltin dilaurate) was used for treatment of 6 species of chicken tapeworms (*Raillietina cesticillus, R. tetragona, Choanotaenia infundibulum, D. proglottina, Hymenolepis carioca,* and *Amoebotaenia sphenoides*) (3). Thus, all efforts toward control must be directed toward prevention by reducing populations of the intermediate hosts.

Trematodes

Trematodes (flukes) are flat, leaflike, parasitic organisms belonging to the phylum Platyhelminthes, class Trematoda. They differ from the cestodes (class Cestoda) in having a digestive system, and they do not form proglottids. The life cycle of all trematodes parasitizing birds requires a molluscan as an intermediate host; some species also use a second intermediate host. Because adult trematodes and larval metacercariae invade almost every cavity and tissue of birds, they may show up unexpectedly at necropsy.

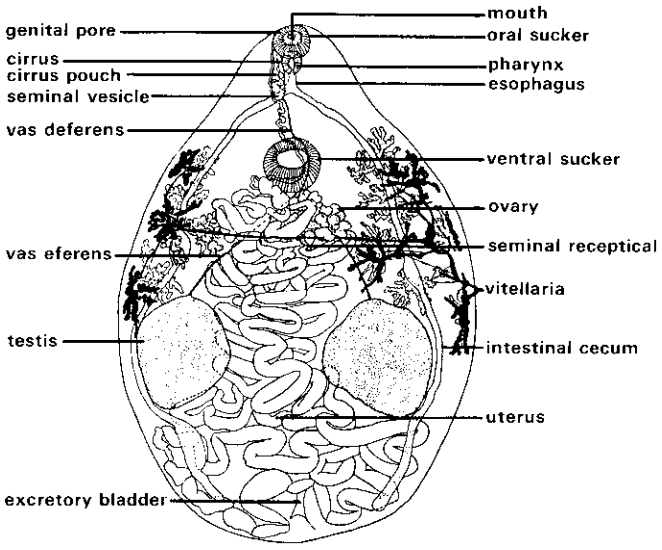
More than 500 species belonging to some 125 genera and 27 families are known to occur in the 4 orders of birds most likely to be submitted to diagnostic laboratories as domestic or pet birds (4). Twenty of these flukes are considered potentially dangerous to poultry in the Western Hemisphere. These flukes belong to 4 orders: Anseriformes (ducks and geese), Galliformes (chickens and turkeys), Columbiformes (pigeons and allies), and Passeriformes (perching birds). Flukes are less host specific than tapeworms, so wild birds often introduce infection in areas where domestic poultry is reared. Because many snails live in ponds and streams, ducks and geese are the most frequently parasitized. The oviduct fluke (*Prosthogonimus* sp.), which is a frequent parasite of many species of wild birds, sometimes causes problems with ducks and chickens (6). This species will be used to illustrate fluke morphology and life history. *P. macrorchis* is the species name recognized in the United States, and this fluke is known as *P. ovatus* or by other specific names in other countries.

Morphology and Life History

The body of the adult fluke (Fig. 27.41) is a flattened oval, and it bears 2 suckers. The digestive system consists of the mouth (within the oral sucker), the pharynx, a short esophagus, and 2 intestinal ceca. An anus is lacking in the trematodes. Two testes and one ovary are present in the same individual. After fertilization, the zygote is enclosed along with yolk cells from the vitellaria by an eggshell. Large numbers of eggs are stored in a prominent convoluted uterus. The excretory system, which originates in a series of flame cells bearing a tuft of cilia, drains with a series of collecting tubules that empty through an excretory pore near the posterior end of the parasite. The arrangement pattern of these collecting tubules is used as a family characteristic in the classification of flukes.

Life Cycle

Adult flukes continually shed eggs, which pass out with the feces of the host. These eggs contain an embryo that develops into a larval stage known as a miracidium. In this group of trematodes, the miracidium hatches after the egg is swallowed by a susceptible snail. Larval development continues within the snail through a succession of stages known as sporocysts and cercariae. The cercariae emerge from the snail and swim about in a lake or pond. Some are drawn into the brachial basket of a dragonfly naiad. The cercaria encysts (metacercaria) and remains in the insect until either the naiad or an infected adult dragonfly is eaten by a bird (Fig. 27.42).



27.41. Morphology of an adult trematode (*Prosthogonimus macrorchis*) (Macy).

Identification

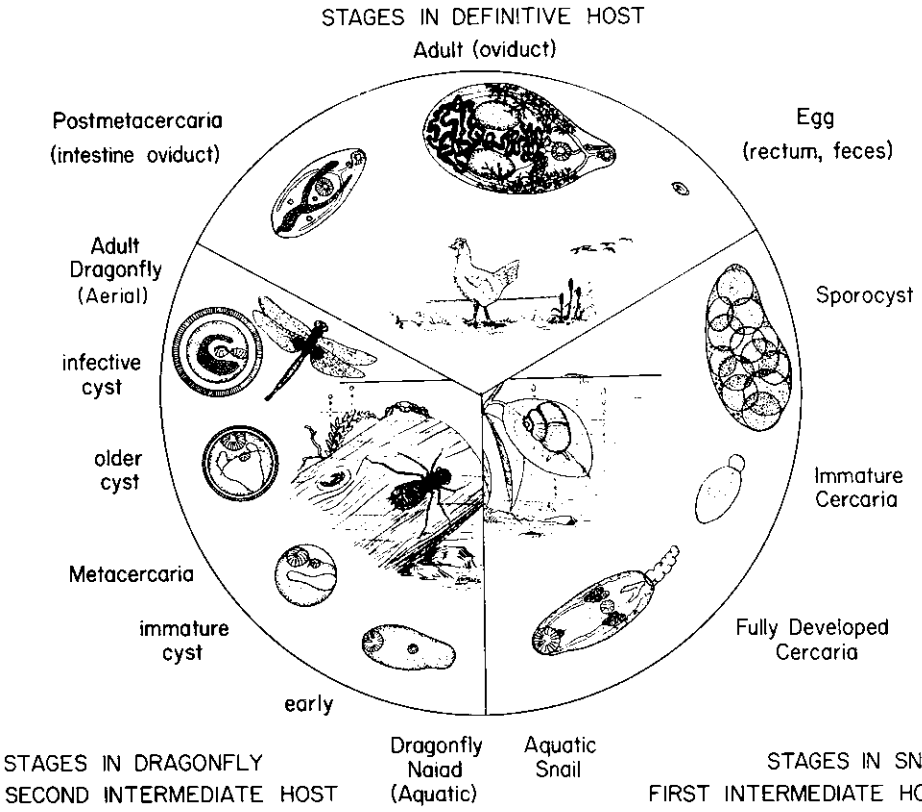
Twenty-four trematodes that are occasionally seen in birds at diagnostic laboratories have been described with keys by Kingston (4). More extensive listings of species are provided by Yamaguti (17), McDonald (7), and Schell (13). The latter text also describes methods of identifying, collecting, preserving, and staining trematodes with emphasis on North American families and genera.

Pathogenicity

Prosthogonimus sp., popularly known as the oviduct fluke, has caused economic losses to poultry producers by 1) drastically reducing egg production after a recent infection and 2) occasionally being enveloped within a hen's egg and later discovered by a complaining customer. Other organs of the bird invaded by flukes include 1) metacercarial cysts in the skin of chickens and turkeys (*Collyriclum faba*); 2) small adult flukes in the conjunctival sac of the eye (*Philophthalmus gralli*); 3) adults in the liver, pancreas, and bile duct of ducks and turkeys (*Amphimerus elongatus*); 4) adults in the collecting tubules of the excretory system of chickens, turkeys, and pigeons (*Tanaisia bragai*); 5) adults and eggs in the circulatory system of ducks by three species of blood fluke; and 6) 14 species of flukes that invade various areas of the digestive tract.

Control

If the life cycle is known and evidence of pathogenicity or economic loss is clear, changes in management are indicated. Efforts should be directed toward fencing poultry off from access to



27.42. Life cycle of a typical digenetic trematode (*P. macrorchis*) (Macy).

lakes or streams where dragonfly naiads, snails and other aquatic intermediate hosts are abundant (6).

No chemotherapeutic products are available for use in poultry, for control or prevention of trematode infections.

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Chapter 28

Protozoal Infections

Introduction

Larry R. McDougald

Protozoal diseases are common in poultry and other birds, and some cause moderate or severe losses. Parasitic diseases differ from viral and bacterial diseases by their eukaryotic nature and often-complicated life cycles. Many parasites utilize intermediate hosts.

Confinement rearing and high-density flocks in the rearing of commercial poultry have increased the exposure to diseases such as coccidiosis and cryptosporidosis, which have short, direct life cycles. In contrast, parasitic diseases that depend on an intermediate host for transmission, such as flukes, many cestodes, and some nematodes, have been practically eliminated from commercial flocks because the specific invertebrates used as hosts are not present in poultry houses. Blackhead disease, caused by the protozoan *Histomonas meleagridis,* can be transmitted directly from bird to bird within a turkey flock, and may utilize intermediate hosts to reach the next susceptible flock.

Control of parasites is largely by treatment or prevention through the use of chemotherapeutic agents. Only with coccidiosis has a good measure of success been achieved by vaccination. The program most widely practiced in control of coccidiosis is continuous administration of anticoccidial drugs in the feed. Such programs allowed better uniformity of treatment and centralized decisions on the choice of programs. This system has proved more reliable than on-farm practices and is used in most commercial poultry flocks today. Success of antiparasitic products may be affected by drug resistance, toxicity from misuse, and a limited spectrum of activity. Despite universal acceptance of this practice, the poultry industry has been under intense pressure to reduce the reliance on chemotherapy. Faced with rising costs, the pharmaceutical industry has shown little interest in developing new products for antiparasitic use, making it unlikely that we will have replacements for the products already available. Immunization against coccidiosis with live vaccines has become more reliable and effective with recent improvements in administration techniques and vaccine strain development. Actions by the Food and Drug Administration (removing the nitroimidazoles from use in animals) have left us with no chemotherapy for treatment of blackhead disease (histomoniasis) in chickens and turkeys. It is ironic that this action has occurred at a time when widespread outbreaks of histomoniasis are causing severe clinical disease in broiler breeder pullets and in turkeys. We are left with no tools other than management or indirect treatment for prevention and control of this disease. As long as highly effective products were available for treatment of outbreaks, research on blackhead disease languished. In the absence of effective treatments, considerable new research has begun, so that we may eventually have vaccines or other treatments for histomoniasis.

Effective control of parasitic diseases depends on accurate diagnosis of the species involved and the extent to which a flock is affected. Serologic methods are rarely used in diagnosis, because the organisms and/or the gross lesions produced by many species are distinctive. Disinfection and quarantine have been of little use in control of coccidiosis and some other parsitic diseases.

Diagnosis is done by gross and microscopic examination of birds taken from a flock for necropsy, or by microscopic examination of feces or tracheal swabs of live birds. Although feasible, serological tests have not been developed for general use. ELISA, Western blot, and other tests are commonly applied in research. PCR testing is now feasible for coccidiosis and histomoniasis, but has been applied only on a research basis.

Protozoa were historically placed in a single phylum, containing all one-celled animals. The complex organization and vastly different structure of protozoa led to the separation of various classes into 7 different phyla (1). Two of these phyla contain species that are important parasites of poultry: The phylum Apicomplexa is characterized by the presence of an apical complex in sporozoites, and all are intracellular parasites. Genera of poultry parasites in this phylum include *Eimeria, Isospora, Haemoproteus, Leucocytozoon, Plasmodium, Toxoplasma, Sarcocystis, Wenyonella, Tyzzeria,* and *Cryptosporidium.*

The second phylum, Sarcomastigophora, includes the flagellates and amebas. Generally, they possess pseudopodia, flagella, or both as locomotor organelles. Genera in this phylum, which are important to poultry, include *Histomonas, Trypanosoma, Chilomastix, Entamoeba, Endolimax,* and *Spironucleus.* The discovery of *Cochlosoma* associated with losses in turkeys and ducks suggests yet another emerging parasitic disease (2, 3).

Encephalitozoon cuniculi, a protozoan in a third phylum, Microspora, recently has been discovered infecting chickens and other birds. This protozoan is egg-transmitted. Infection can be associated with embryo mortality but is usually unapparent. Affected birds may show inactivity, lameness, mild diarrhea, and weight loss. Parasites have been identified in the digestive tract,

urogenital organs, and muscle. In embryos, brain and heart also were found to be infected (4, 5). It is not known how widespread or important microsporidians may be in poultry.

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Coccidiosis

Larry R. McDougald and Steve H. Fitz-Coy

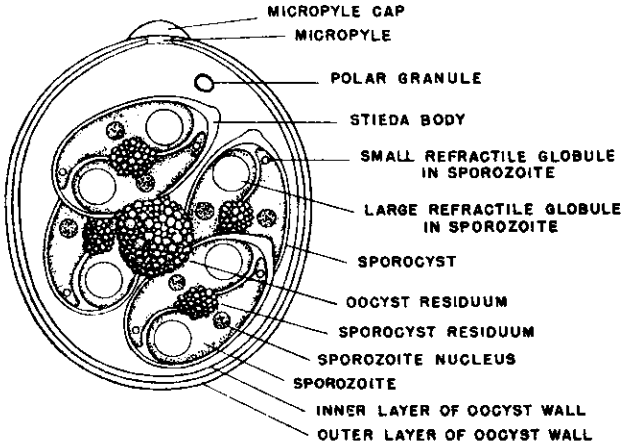
Introduction

Coccidiosis is a disease of universal importance in poultry production. The protozoan parasites of the genus *Eimeria* multiply in the intestinal tract and cause tissue damage, with resulting interruption of feeding and digestive processes or nutrient absorption, dehydration, blood loss, loss of skin pigmentation and increased susceptibility to other disease agents. Historically, the spectacular onset of coccidiosis with bloody diarrhea and high mortality inspired awe and dread on the part of poultry growers and bird fanciers. Like many parasitic diseases, coccidiosis is largely a disease of young animals because immunity quickly develops after exposure and gives protection against later disease outbreaks. Unfortunately, no cross-immunity exists between species of *Eimeria* in birds, and later outbreaks may be the result of different species. The short, direct life cycle and high reproductive potential of coccidia in poultry often lead to severe outbreaks of disease in small backyard flocks or in the modern poultry house, where 15–30,000 chickens may be reared on litter.

Coccidiosis may strike any type of poultry in any type of facility. The disease may be mild, resulting from the ingestion of a few oocysts, and may escape notice, or it may be severe as a result of ingestion of millions of oocysts. Most infections are relatively mild, but because of the potential for the disastrous outbreak and the resulting financial loss, almost all young poultry are given continuous medication with low levels of anticoccidial drugs, which prevent the infection or reduce infections to a low, immunizing level. Vaccines against coccidiosis have in the past been used mostly in breeder pullets and in turkeys. Vaccination of broilers has rarely been practiced because even light infections with some species of coccidia can affect weight gain, feed conversion, and pigmentation of the skin. However, new vaccines with improved administration techniques are targeting this larger market with encouraging results.

Classification and Taxonomic Relationships

The biology and taxonomy of coccidia were reviewed by Long (24) and Pellerdy (37). Coccidia are members of the phylum Apicomplexa, which is characterized by the presence of an apical complex in sporozoites. All apicomplexans are intracellular parasites. The genera *Eimeria, Isospora, Haemoproteus, Leucocytozoon, Plasmodium, Toxoplasma, Sarcocystis, Wenyonella, Tyzzeria,* and *Cryptosporidium* are found in poultry.



28.1. Diagram of sporulated oocyst of genus *Eimeria*. The oocysts of all *Eimeria* contain 4 sporocysts, each with 2 sporozoites, after sporulation.

The most common apicomplexans in poultry belong to the genus *Eimeria* described in this section or the genus *Cryptosporidium* discussed in a later section of this chapter. The oocyst, a thick-walled zygote shed in fecal matter by the infected host, is fairly distinctive and is often used in diagnosis and identification of species. Oocysts are enclosed in a thick outer shell and consist of a single cell that begins the process of sporulation to yield the infective stage in 48–72 hours. Infective oocysts contain 4 sporocysts, which in turn contain 2 sporozoites (Fig. 28.1).

The closely related parasites, *Sarcocystis*, *Toxoplasma, Cryptosporidia*, and avian malaria, are discussed in the sections "Miscellaneous and Sporadic protozoal infections" and "Cryptosporidiosis."

When oocysts are ingested, the oocyst wall is crushed in the gizzard, and the sporozoites are released from sporocysts by the action of chymotrypsin and bile salts in the small intestine. Sporozoites enter epithelial cells or are taken into intraepithelial lymphocytes, where development begins. Species of coccidia are identified on the basis of 1) oocyst morphology, 2) host specificity, 3) immune specificity, 4) appearance and location of gross lesions within the natural host, and 5) length of the prepatent pe-

riod. The host specificity of *Eimeria* in birds and mammals is very strict, so that parasites from different species of birds or animals can be considered different species, even though they may have similar-appearing oocysts.

Biological characteristics useful in the identification of species are 1) location of lesions in the intestine; 2) appearance of gross lesions; 3) oocyst size, shape and color; 4) size of endogenous tissue stages (schizonts, merozoites, meronts, gametocytes); 5) location of the parasites within tissues; 6) minimum prepatent period in experimental infections; and 7) immunogenicity in comparison with reference strains. In recent years biochemical and molecular tools have also been used for identification of coccidia. Techniques of value include the electrophoretic pattern of metabolic enzymes (40) and PCR (42). Monoclonal antibodies are useful in experimental work but have not been developed to be specific for species identification. Digital image analysis (6) is useful for analysis of photographic imagery. For diagnostic purposes, the traditional biological characteristics are usually adequate. However, taxonomic difficulties are encountered in in identification of species with morphologically similar oocysts which are found with overlapping tissue specificity. Species can be identified by comparison of isolates with several criteria listed in Table 28.2 or 28.3.

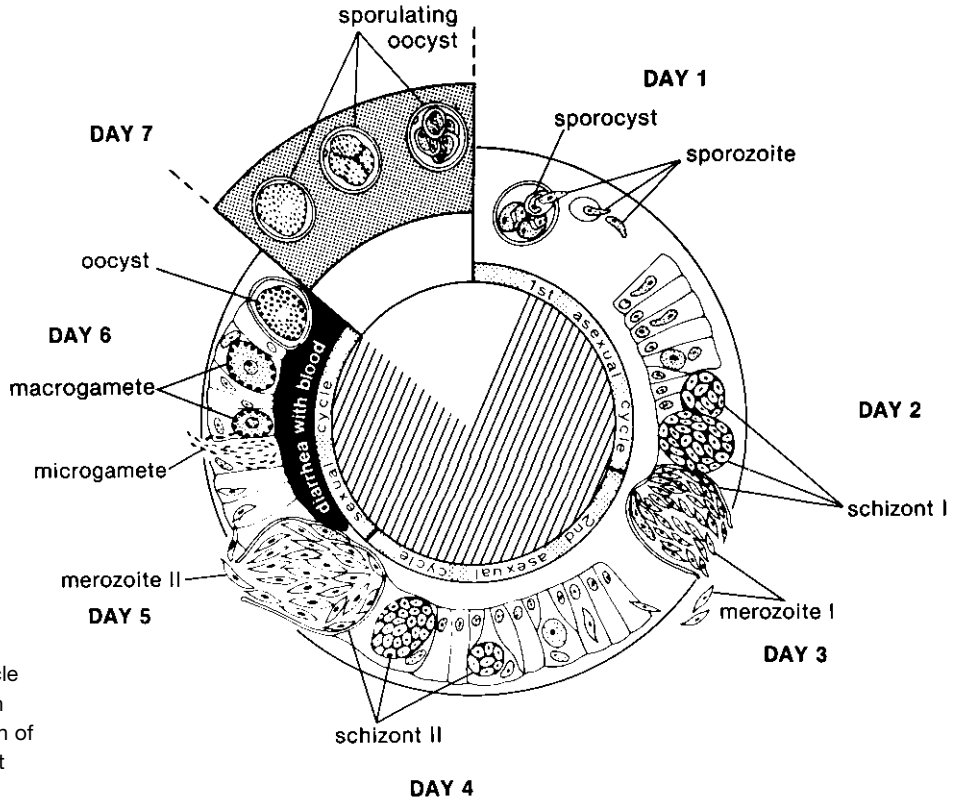
Life Cycle

Coccidiosis differs from bacterial and viral diseases in the self-limiting nature of its development. The life cycle of *E. tenella* (Fig. 28.2) is typical of all *Eimeria,* although some species vary in the number of asexual generations and the time required for each developmental stage. After the oocyst wall is crushed in the gizzard and the sporozoites are released, the sporozoites enter cells in the mucosa of the intestine and begin the cell cycle leading to reproduction. At least 2 generations of asexual development (sometimes as many as 4) called schizogony or merogony give rise to a sexual phase, where small, motile microgametes seek out and unite with macrogametes. The resulting zygote matures into an oocyst, which is released from the intestinal mucosa and is shed in the feces. With each species, the reproductive potential from a single ingested oocyst is fairly constant. The entire process takes 4–6 days, depending on species, although oocysts may be shed for several days after patency is reached. In some species (*E. tenella, E. necatrix*), the maximum tissue damage may occur when second-generation schizonts rupture to release merozoites. Other species may have small scattered schizonts, which cause little damage, but the gametocytes may elicit a strong reaction with cellular infiltration and thickened, inflamed tissues.

Relationship between Coccidiosis and Other Poultry Diseases

The tissue damage and changes in intestinal tract function may allow colonization by various harmful bacteria, such as *Clostridium perfringens*, leading to necrotic enteritis (17, 26), or *Salmonella typhimurium* (2, 3). Cecal coccidiosis (*E. tenella*) may contribute to increased severity of the blackhead organism (*Histomonas meleagridis*) in chickens. Experimental infections with the 2 organisms were characterized by a higher incidence of hepatic disease, as compared with monoinfection with *Histomonas* (28).

Immunosuppressive diseases may act in concert with coccidiosis to produce a more severe disease. Marek's disease may interfere with development of immunity to coccidiosis (4), and infec-



28.2. The 7-day life cycle of *E. tenella* includes 2 or more asexual and 1 sexual cycle during the 6 days after an oocyst has been swallowed by the host. The new generation of oocysts becomes infective to the next host after sporulation.

tious bursal disease (IBD) may exacerbate coccidiosis, placing a heavier burden on anticoccidial drugs (29).

Coccidiosis in Chickens

Coccidiosis remains one of the most expensive and common diseases of poultry production in spite of advances in chemotherapy, management, nutrition, and genetics. The disease is often diagnosed in birds brought to diagnostic laboratories (1), but the vast majority of cases are diagnosed in the field and handled by poultry service personnel. The current expense for preventive medication exceeds $90 million in the United States and more than $300 million worldwide.

Incidence and Distribution

Coccidia are found wherever chickens are raised. Their strict host specificity eliminates wild birds as sources of infection. The most common means of spread of coccidia is mechanical, by personnel who move between pens, houses, or farms. Coccidial infections are self-limiting and depend largely on the number of oocysts ingested and on the immune status of the bird. Surveys in North and South America revealed coccidia present in almost all broiler farms (25, 30, 32). Very high percentages of positive flocks were also reported from Europe (5, 23, 43). Oocysts in the litter or droppings of broiler chickens are usually most numerous between 3 and 5 weeks of age and often decline thereafter. Few oocysts are found after birds are removed from a farm, because the parasites are killed by ammonia or composting heat in poultry litter or droppings. The ubiquitous nature of poultry coccidia precludes the possibility of elimination or prevention of coccidia by quarantine, disinfection, or sanitation.

Etiology and Diagnosis

Nine species of *Eimeria* have been described from chickens (Table 28.1). New evidence suggests that all should be considered valid. Concurrent infection with 2 or more species of coccidia is common (31). Each species causes separate and distinct, recognizable diseases, independent of the other species.

Characteristics useful in the identification of species are as follows: 1) location of the lesions in the intestine; 2) appearance of the gross lesion; 3) oocyst size, shape, and color; 4) size of schizonts and merozoites; 5) location of parasites in tissues (type of cell parasitized); 6) minimum prepatent period in experimental infections; and 7) immunogenicity against reference strains. In recent years, more emphasis has been placed on biochemical and physiologic identification of coccidia. Promising new tools for species identification include electrophoresis of metabolic enzymes (41) and PCR (42). Monoclonal antibodies are useful in experimental work but have not been suitably specific to distinguish species, probably because of common antigens. For diagnostic purposes, the traditional characteristics are adequate, and a satisfactory diagnosis can be made from Table 28.2. Cross-immunity and biochemical studies require pure species isolates propagated from single oocysts. The severity of infection based on gross lesions is often graded on a scale of 0–4 as described by Johnson and Reid (22), where 0 is normal and 4 is the maximum lesion. Microscopic examination of lesions or droppings is also used to grade infections, by counting the number of parasite forms in a field. In this techinque, at least 5 fields should be examined. A typical scoring system used by some diagnosticians is based on the species, number, and type of parasites. For *E. maxima* a typical score system is: 0 = no parasites, 1 = 1–10 parasites/field, 2 = 11–20, 3 = 21–49 and 4 = 50 or more/field. With other species, 1 = 1–25/field, 2 = 26–50, 3 = 51–75 and 4 = greater than 75.

Eimeria acervulina *Tyzzer 1929*

This species is the most frequently encountered in commercial poultry in North and South America (30, 31, 32) and is commonly reported in other continents. Oocysts are ovoid and often show thinning of the shell at the small end. The average size of oocysts is 18.3 × 14.6 μm, but the range is 17.7–20.2 × 13.7–16.3 μm.

Pathogenicity

Severity of infection may vary with the isolate, the number of oocysts ingested, and the immune state of the bird. Ingestion of 10^3, 10^4, 10^5, or 10^6 oocysts by young white rock chicks resulted in mild to severe coccidiosis, with lesion scores ranging from 1.1 (10^3 oocysts) to 4.0 (10^6 oocysts) (38). Reduction in rate of weight gain was also proportional to the infective dose. Watery and mucoid droppings may be seen as early as 4 days post-exposure. Heavy infections often cause lesions to coalesce, and sometimes mortality may result. Light to moderate infections may produce little effect on weight gain and feed conversion but may cause loss of carotenoid and xanthophyll pigments from the blood and skin because of reduced absorption in the small intestine. The intestinal mucosa may be thickened, resulting in poor feed conversion. Egg production may be depressed in laying birds.

Gross White Plaques, Arranged Transversely

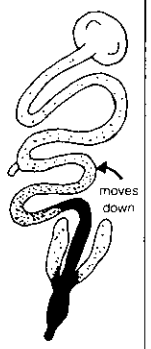
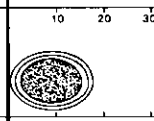
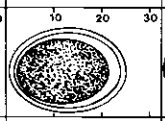
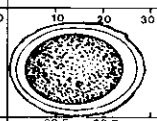
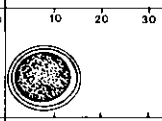
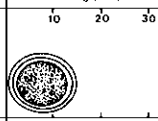
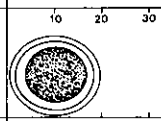
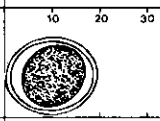
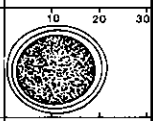
The intestine may be pale and contain watery and mucoid fluid. The gross lesion in light infections is limited to the duodenal loop, with only a few plaques/cm. In heavy infections, lesions may extend some distance through the small intestine, and plaques may overlap or coalesce. The plaques are generally smaller in heavy infections due to crowding. The lesions may be composed of schizonts, gametocytes, and developing oocysts. Microscopy of smears from intestinal lesions usually reveals numerous oocysts and gametocytes of varying stage of development.

Histopathology of the small intestine reveals the ovoid gametocytes in the mucosal cells lining the villi. In moderate to heavy infections, the tips of villi are broken off, leading to truncation and fusion of villi and thickening of the mucosa. Some epithelial cells may contain more than one parasite. Capillaries may be engorged with red blood cells and there is infiltration of granulocytes in the area parasitized. Schiff's reagent will stain the macrogametes and developing oocysts a brilliant red, because of the polysaccharide used in oocyst wall formation.

Eimeria brunetti *Levine 1942*

About 10–20% of field isolates in surveys in the United States and South America contained *E. brunetti* (25, 30, 31). The oocysts of *E. brunetti* average 24.6 × 18.8 μm and are easily confused with *E. tenella.* This species is found primarily in the lower

Table 28.1. Diagnostic table of coccidia

	CHARACTERISTICS	E. acervulina	E. brunetti	E. maxima	E. mitis †	E. mivati ‡	E. necatrix	E. praecox	E. tenella	SPECIES OF DOUBTFUL VALIDITY E. hagani
MACROSCOPIC LESIONS	ZONE PARASITIZED									
	MACROSCOPIC LESIONS	light infection: whitish round lesions sometimes in ladder-like streaks heavy infection: plaques coalescing, thickened intestinal wall	coagulation necrosis mucoid, bloody enteritis in lower intestine	thickened walls, mucoid, blood - tinged exudate, petechiae	no discrete lesions in intestine, mucoid exudate	light infection rounded plaques of oocysts heavy infection thickened walls coalescing plaques	ballooning, white spots (schizonts), petechiae, mucoid blood - filled exudate	no lesions, mucoid exudate	onset: hemorrhage into lumen later: thickening, whitish mucosa, cores clotted blood	pinhead hemorrhages petechiae
MICROSCOPIC CHARACTERISTICS	MILLIMICRONS OOCYSTS REDRAWN FROM ORIGINALS									non available
	LENGTH x WIDTH μ LENGTH = WIDTH =	AV = 18.3 x 14.6 17.7 - 20.2 13.7 - 16.3	24.6 x 18.8 20.7 - 30.3 18.1 - 24.2	30.5 x 20.7 21.5 - 42.5 16.5 - 29.8	15.6 x 14.2 11.7 - 18.7 11.0 - 18.0	15.6 x 13.4 11.1 - 19.9 10.5 - 16.2	20.4 x 17.2 13.2 - 22.7 11.3 - 18.3	21.3 x 17.1 19.8 - 24.7 15.7 - 19.8	22.0 x 19.0 19.5 - 26.0 16.5 - 22.8	19.1 x 17.6 15.8 - 20.9 14.3 - 19.5
	OOCYST SHAPE AND INDEX-LENGTH/WIDTH	ovoid 1.25	ovoid 1.31	ovoid 1.47	subspherical 1.09	ellipsoid to broadly ovoid 1.16	oblong ovoid 1.19	ovoidal 1.24	ovoid 1.16	broadly ovoid 1.08
	SCHIZONT, MAX IN MICRONS	10.3	30.0	9.4	15.1	17.3	65.9	20	54.0	
	PARASITE LOCATION IN TISSUE SECTIONS	epithelial	2nd generation schizonts subepithelial	gametocytes subepithelial	epithelial	epithelial	2nd generation schizonts subepithelial	epithelial	2nd generation schizonts subepithelial	epithelial
LIFE HISTORY CHARACTERISTICS	MINIMUM PREPATENT PERIOD-HR	97	120	121	93	93	138	83	115	99
	SPORULATION TIME MINIMUM (HR)	17	18	30	15	12	18	12	18	18

† = From Norton and Joyner (1980)
‡ = As described by Edgar and Siebold (1964)
◉ = Compiled from various sources (1982)

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Table 28.2. Preventive anticoccidials approved by FDA for use in feed formulation. (Historical and scientific interest only. Not all products are available.) (9)

Trade or Empirical Name, Approval Label (Manufacturer)	Trade Name	First Approval by FDA	Drug Withdrawal (Days before Slaughter)
Sulfaquinoxaline, 0.015–0.025% (Merck)	SQ, Sulquin	1948	10
Nitrofurazone, 0.0055% (Hess & Clark; Smith-Kline)	nfz, Amifur	1948	5
Arsanilic acid or sodium arsanilate, 0.04% for 8 days (Abbott)	Pro-Gen	1949	5
Butynorate, 0.0375% for turkeys (Solvay)	Tinostat	1954	28
Nicarbazin, 0.0125% (Merck)	Nicarb	1955	4
Furazolidone, 0.0055–0.011% (Hess & Clark)	nf-180	1957	5
Nitromide, 0.025% + sulfanitran, 0.03% + roxarasone, 0.005% (Solvay)	Unistat-3	1958	5
Oxytetracycline, 0.022% (Pfizer)	Terramycin	1959	3
Amprolium, 0.0125–0.025% (MSD-AGVET)	Amprol	1960	0
Chlortetracycline, 0.022%	(American Aureomycin Cyanamid)	1960	(See feeding restrictions)
Zoalene, 0.004–0.0125% (Solvay)	Zoamix	1960	(higher levels, 5 days)
Amprolium, 0.0125% + ethopabate, 0.0004/0.004% (Merck)	Amprol Plus, Amprol Hi-E	1963	0
Buquinolate, 0.00825% (Norwich-Eaton)	Bonaid	1967	0
Clopidol or meticlorpindol, 0.0125–0.025% (A. L. Laboratories)	Coyden	1968	0 days at 0.0125%; 5 days at 0.025%
Decoquinate 0.003% (Rhone-Poulenc)	Deccox	1970	0
Sulfadimethoxine, 0.0125% + ormetoprim, 0.0075% (Hoffmann-La Roche)	Rofenaid	1970	5
Monensin, 0.01–0.0121 % (Elanco)	Coban	1971	0
Robenidine, 0.0033% (American Cyanamid)	Robenz, Cycostat	1972	5
Lasalocid, 0.0075–0.0125% (Hoffmann-La Roche)	Avatec	1976	3
Salinomycin, 0.004–0.0066% (Agri-Bio)	Bio-Cox	1983	0
Halofuginone, 3 ppm (Hoechst-Roussell Agri-Vet)	Stenorol	1987	5
Narasin, 54–72g/T (Elanco)	Monteban	1988	0
Madurimicin, 5–6 ppm (American Cyanamid)	Cygro	1989	5
Narasin + nicarbazin,54–90 g/T (Elanco)	Maxiban	1989	5
Semduramycin, 25ppm (Pfizer)	Aviax	1995	0
Diclazuril, 1 ppm (Schering-Plough)	Clinicox	1999	0

small intestine, usually from the yolk sac diverticulum to near the cecal juncture. In severe cases, the lesion may extend from the gizzard to the cloaca and extend into the ceca (Fig. 28.3 E–H). Most field infections are difficult to recognize based on gross lesions but can be confirmed by observation of typically sized oocysts by microscopy. The average oocyst size is 24.6 × 18.8 μm, with a range of 20.7–30.3 by 18.1–24.2 μm. Oocysts are ovoid, with a length/width index of 1.31.

Pathogenicity

Although less serious than *E. tenella* or *E. necatrix, E. brunetti* can produce moderate mortality, loss of weight gain, poor feed conversion, and other complications. Inoculation with 1–2 × 10^5 oocysts frequently will cause 10–30% mortality and reduced gain in survivors. Light infections of *E. brunetti* are overlooked easily unless careful attention is paid to the lower small intestine. Such light infections can cause reduced weight gain and poor feed conversion, even though gross lesions are not clearly apparent.

Gross Lesions and Histopathology

At early stages of infection, the mucosa of the lower small intestine may be covered with tiny petechiae and have some thickening, loss of color and watery contents. In heavy infections, the mucosa is badly damaged, with coagulation necrosis appearing on days 5–7 postinfection (PI) and with a caseous eroded surface over the entire mucosa. Coagulated blood and mucosal casts will be apparent in the droppings. Thickening of the mucosa and edematous swelling occurs in severe infections, especially on the sixth day PI.

The asexual stages of first- and second-generation schizogony generally occur in the upper small intestine. Histopathology on the fourth day of infection reveals schizonts, cellular infiltration, and some damage to the mucosa. By the fifth day, many of the tips of villi are broken off. Merozoites invade the epithelium and develop into sexual stages in the lower small intestine and ceca. In severe cases, the villi may be completely denuded, leaving only the basement membranes intact.

Eimeria hagani *Levine 1938*

The taxonomic status of *E. hagani* was considered by some to be in doubt because the original description was incomplete. However, a strain of *E. hagani* was studied in depth by Oluleye (36). The oocysts average 18.0 × 14.7 μm (sporulated 19.6 × 14.7 μm). Sporocysts are 11.3 4× 6.9 mm, and sporozoites are

12.9 × 2.1 μm. The prepatent period is 98 hrs. Sporulation requires 17–44 hrs at 23.5°C. The gross lesions consist of petechiae and white opacities in the upper small intestine. There is not blood, although the mucosa can appear reddish. The intestinal contents may be creamy or watery. In histopathology, the parasites are seen in the tips of villi, some as far as 2/3 down the length. Maturation of first generation schizonts is 36–48 hrs, second generation at 60 hrs, and third generation by 96 hrs. The schizonts average 14.4 × 13.2, 6.2 × 5.8, or 8.9 × 3.1 μm for first, second- or third-generation schizonts, respectively. Merozoites were 8.8 × 1.1 mm. Immunity is species specific, separating it from *E. acervulina, E. mitis, E. mivati,* and other species. This species is described as producing hemorrhagic spots, catarrhal inflamation, engorged capillary beds and watery intestinal contents between 96 and 120 hr PI, and is moderately pathogenic.

Eimeria maxima *Tyzzer 1929*

The mid-small intestine is often parasitized with *E. maxima,* from below the duodenal loop past the yolk sac diverticulum, but in heavy infections, the lesions may extend throughout the small intestine. *E. maxima* is an easy species to recognize because of the characteristic large oocysts, 30.5 × 20.7 mm (21.5–42.5 × 16.5–29.8), which usually have a distinctive yellowish color (Fig. 28.3A,F,G,H,I,J). Oocysts have a shape index of 1.473. An abundance of yellow-orange mucus and fluid often is in the midgut. This species can be differentiated from *E. necatrix* by the lack of large schizonts associated with the lesions and from *E. brunetti* by the larger oocysts and the appearance and location of the lesions.

Pathogenicity

E maxima is moderately-highly pathogenic. Infection with 50–200 × 10^3 oocysts causes poor weight gain, morbidity, diarrhea, and sometimes mortality. Some isolates are capable of 30% mortality in 5-week-old chickens with 100,000 oocysts. There is often extreme emaciation, pallor, roughening of feathers, and anorexia. Producers interested in maintaining good skin color in chickens must be concerned with subclinical infections because of the effect of this species on absorption of xanthophyll and carotenoid pigments in the small intestine.

Gross Lesions and Histopathology

Minimal tissue damage occurs with the first 2 asexual cycles, which develop superficially in the epithelial cells of the mucosa. When the sexual stages develop in deeper tissues on days 5–8 PI, lesions develop because of congestion and edema, cellular infiltration, and thickening of the mucosa. Infected host cells become enlarged, pushing into the subepithelial zone. Microscopic hemorrhages occur near the tips of the villi, and foci of infection can be seen from the serosal surface. The intestine may be flaccid and filled with fluid, and the lumen often contains yellow or orange mucus and blood. This condition has been described as "ballooning." Microscopic pathology is characterized by edema and cellular infiltration, developing schizonts through day 4, and sexual stages (macrogametes and microgametes) in deeper tissues on days 5–8. In severe infections, considerable disruption of the mucosa occurs.

Eimeria mitis *Tyzzer 1929*

The lower small intestine is the normal site of this parasite, from the yolk sac diverticulum to the cecal necks. The lesions are normally indistinct with this species, but the potential for pathogenic effects on weight gain and morbidity is well documented (13). Oocysts average 16.2 × 16.0 (shape index 1.01), giving them a subspherical appearance.

Pathogenicity

Infection with 5 × 10^5 − 5 × 10^6 oocysts will reduce weight gain and cause morbidity and loss of pigmentation in broiler chickens. In layers, this species may affect egg production and induce a molt. The lack of distinct gross lesions causes this species to be overlooked or misdiagnosed in subclinical infections.

Gross Lesions and Histopathology

Clinically, the gross lesion is very slight and can be easily overlooked. The lower small intestine appears pale and flaccid, and microscopic examination of smears from the mucosal surface may reveal numerous tiny oocysts (15.6 × 14.2 mm). The infection is distinguished easily from *E. brunetti* by the smaller, round oocysts. In light infections, the appearance of the gross lesion may be similar to *E. brunetti.* The gross lesions of this species are unremarkable because the developing parasites do not tend to localize in colonies as do other species, and the schizonts and gametocytes are superficial in the mucosa.

Eimeria mivati *Edgar and Siebold 1964*

This species was first identified in 1959 as a small strain of *E. acervulina* and later named as a separate species (11). The zone of infection extends from the duodenal loop to the ceca and cloaca in heavy infections. Oocysts are broadly ovoid, averaging 15.6 × 13.4, and the shape index is 1.16.

Considerable controversy exists on the validity of *E. mivati* as a species, dating from the work of Shirley with isoenzymes and later work with species-specific primers for PCR. However, recent evaluation of field samples have produced isolates that fit the morphologic description of Edgar and Seibold, and do not react with primers for other known species. Results using the ITS1 and ITS2 region primers indice that these organisms are different from the other 7 known species for which specific primers have been developed. Further work is needed to settle the taxonomic status of this species.

Pathogenicity

Infection with 5 × 10^5 – 1 × 10^6 oocysts of *E. mivati* causes reduced weight gain and morbidity. Mortality as high as 40% has been seen in experimental infections (personal observations).

Gross Lesions and Histopathology

Early lesions appear in the duodenum and later in the midgut and lower small intestine. In light infections, isolated lesions resemble those of *E. acervulina* but are more circular in shape. These

lesions, representing colonies of gametocytes and developing oocysts, may be seen from the serosal surface of the gut. Gross lesions sometimes include red petechiae and round white spots for 72–240 hr PI. Histopathology reveals parasitism of the mucosal cells of the villi of the small intestine. In contrast to *E. acervulina,* this species may be found from the tip to the base of villi, sometimes causing severe denuding of the mucosa.

Eimeria necatrix *Johnson 1930*

Because of the spectacular lesions in the small intestine, this species was one of the best known by early poultry producers. The lesion is found in the small intestine in approximately the same location as *E. maxima* (Fig. 28.3A–D). Probably because of the low reproductive capability of *E. necatrix,* it is not able to compete with other coccidia and is diagnosed mostly in older birds such as brooder pullets or layer pullets 9–14 weeks old. The intestine often is dilated to twice its normal size (ballooning), and the lumen may be filled with blood and fluid laden with merozoites and clusters of large mature schizonts. The oocysts are ovoid and average 20.4 × 17.2 mm, which is near in size to those of *E. tenella.* Curiously, the oocysts are found only in the ceca, rather than in the intestine where lesions are found. The sexual stages (gametocytes) develop ceca, and are scattered rather than clustered. *E. necatrix* is a poor producer of oocysts.

Pathogenicity, Gross Lesions, and Histopathology

E. necatrix along with *E. tenella* are the most pathogenic of the chicken coccidia. Infection with 10^4–10^5 oocysts is sufficient to cause severe weight loss, morbidity, and mortality. Survivors may be emaciated, suffer secondary infections, and lose pigmentation. Droppings of infected birds often contain blood, fluid, and mucus. Naturally occurring infections have caused mortality in excess of 25% in commercial flocks. In experimental infections, 100% mortality is possible. Layer pullets suffering outbreaks at 7–20 weeks of age may suffer mortality, morbidity, loss of uniformity, and decreased egg-laying potential. Gross lesions may be seen as early as 2–3 days PI, associated with first-generation schizogony, but the severe lesions at 4–6 days PI are caused by second-generation schizogony. The intestine may be ballooned; the mucosa thickened; and the lumen filled with fluid, blood, and tissue debris. From the serosal surface, the foci of infection can be seen as small white plaques or red petechiae. In dead birds, these lesions appear white and black, giving rise to the expression "salt and pepper" appearance. Smears examined microscopically on days 4–5 PI may contain numerous clusters of large (66 mm) schizonts, each containing hundreds of merozoites. The clusters of schizonts deep in the mucosa often penetrate the submucosa and damage the layers of smooth muscle and destroy blood vessels. In these instances, the foci are large enough to be seen from the serosal surface. Later, scar tissue may be seen where epithelial regeneration is incomplete. Few pathogenic effects are seen with the invasion of the cecal mucosa by the third-generation schizonts and gametocytes because of the nonclustering nature of these stages. The third-generation schizonts produce only 6–16 merozoites.

Lesions may extend from the ventriculus-gizzard junction to the ileo-cecal junction in severe infections, causing dilation (ballooning) and thickening of the mucosa. The lumen may be filled with blood and pieces of mucosal tissue. Microscopic examination of smears from the mucosal surface reveals numerous clusters of large schizonts, which are characteristic for this species and distinguish it from others that overlap in habitat. Also, oocysts are never associated with lesions of this species.

Histopathology of midgut from affected birds reveals a submucosa and lamina propria crowded with large clusters of schizonts. Often, large areas of the mucosa are sloughed off, and the lesion may extend through the muscle layers to near the serosal membranes.

Eimeria praecox *Johnson 1930*

This species is named from the short prepatent period (about 83 hours); hence a "precocious" parasite. Even though *E. praecox* is often overlooked because no prominent lesions exist, it is easily detected by timed infections of experimental birds. The oocysts are recognized easily because they are generally larger than those of other species found in the duodenum. At 21.3 × 17.1 mm, they are larger than *E. acervulina, E. mivati,* and *E. mitis* and smaller than *E. maxima.* The shape index is 1.25.

Pathogenicity, Gross Lesions, and Histopathology

Heavy infections cause reduced weight gain, loss of pigmentation, dehydration, and poor feed conversion. The gross lesions consist of watery intestinal contents and sometimes mucus and mucoid casts. Most of the infection is confined to the duodenal loop. Small pinpoint hemorrhages may be seen on the mucosal surface on days 4–5 of infection. Recent studies suggest that this species may cause morbidity and reduced weight gain (15). Severe infections may cause dehydration. The epithelial cells of the sides of the villi (but not the tips) are most often infected. There may be several parasites in each cell. Three to four asexual generations are normal, followed by gametogony. Infections with this species cause little tissue reaction.

Eimeria tenella *(Railliet and Lucet 1891) Fantham 1909*

E. tenella is the best known of poultry coccidia, because of the easily recognizable lesions and often spectacular losses it causes in commercial broilers or layer pullets. This species inhabits the ceca (rarely adjacent intestinal tissues), causing a severe disease characterized by bleeding, high morbidity and mortality, lost weight gain, emaciation, loss of skin pigmentation, and other signs. Oocysts are ovoid, averaging 22.0 × 19.0 mm (shape index 1.16). Diagnosis is dependent upon finding cecal lesions with prominent blood and often firm bloody cores and accompanying clusters of large schizonts and oocysts (Fig. 28.3I–L).

Pathogenicity, Pathogenesis, and Epidemiology

Experimental inoculation with 10^4 or more sporulated oocysts can cause morbidity, mortality, and greatly reduced weight gain, making this one of the most pathogenic species in chickens. Inoculation with 10^3 oocysts is sufficient to cause bloody droppings and other signs of infection. The most pathogenic stage is

the second-generation schizont, which matures at 4 days PI. Like *E. necatrix,* this species produces clusters of large schizonts, which may contain hundreds of merozoites. The schizonts develop deep in the lamina propria, so that the mucosa and associated blood vessels are disrupted when the schizonts mature and merozoites are released. Onset of mortality in a flock is rapid. Most of the mortality occurs between days 5 and 6 PI, and in acute infections, it may follow the first signs of infection by only a few hours. Blood loss may reduce the erythrocyte count and hematocrit value as much as 50%. The maximum effect on weight gain is seen at 7 days PI. Some of the weight lost from dehydration may be regained quickly, but growth will always lag behind that of uninfected birds. The exact cause of death is not known, but toxic factors are suspected. Blood loss alone does not account for mortality. In a few cases, death may result from gangrenous or ruptured cecal pouches. Extracts of infected cecal pouches produce acute blood coagulation and death when injected intravenously into other chicks. The possible role of bacterial products in mortality from coccidiosis is suggested by the lack of mortality from *E. tenella* in germ-free chicks.

Gross Lesions and Histopathology

Even during maturation of the first generation of schizonts, small foci of denuded epithelium may be seen. By day 4 PI, the second-generation schizonts are maturing, and hemorrhages are apparent. The cecal pouch may become greatly enlarged and distended with clotted blood and pieces of cecal mucosa in the lumen. On days 6 and 7, the cecal core becomes hardened and drier; eventually it is passed in the feces. Regeneration of the epithelium is rapid and may be complete by day 10. The infection usually can be seen from the serosal surface of the ceca as dark petechiae and foci, which become coalesced in severe infections. The cecal wall is often greatly thickened because of edema and infiltration and later scar tissue.

Microscopically, the first-generation schizonts are widely scattered and mature at 2–3 days PI. Small focal areas of hemorrhage and necrosis may appear near blood vessels of the inner circular muscles of the muscularis layer. Heterophil infiltration of the submucosa proceeds rapidly as the large second-generation schizonts develop in the lamina propria. These are found in clusters or colonies that generally are progeny of a single first-generation schizont. Maturation of the second-generation schizonts is accompanied by excessive tissue damage, bleeding, disruption of the cecal glands, and destruction of the mucosa and muscularis layer. Microgametes and macrogametes are seen in the tissues on days 6 and 7, and mature oocysts are released into the lumen in large numbers. Regeneration of the epithelium and glands may be complete by day 10 in light infections, but the epithelium may never completely recover in severe infections. Lost muscularis mucosa is not replaced, and the submucosa becomes densely fibrosed.

Epidemiology of Coccidiosis

Natural and Experimental Hosts

The chicken is the only natural host of these 7 species of *Eimeria.* Reports of these species of *Eimeria* infecting other birds can be considered spurious. Cross-transmission of *Eimeria* spp. from chickens to other host species has been unsuccessful except for a few instances in which severely immunocompromised birds were used.

Naive chickens of all ages and breeds are susceptible to infection. However, immunity develops after mild infections, limiting further infection. Newly hatched chicks often have high levels of maternal antibodies but it does not appear that this limits susceptibility. Outbreaks are common at 3–6 weeks of age and are seldom seen in poultry flocks at less than 3 weeks. In special situations, infections may be seen as early as 1 week of age. Data from routine necropsy of chickens over several years in the USA (Personal observations of S. Fitz-Coy, based on gross and microscopic evidence) showed that the prominent species were *E. acervulina* (97%), *E. maxima* (64%), and *E. tenella* (64%). Less common species were *E. mivati, E. brunetti, E. mitis* and *E. praecox.* Surveys of coccidia in broiler houses in Georgia demonstrated that oocysts of coccidia build up during the growth of a flock and then decline as the birds become immune to further infection (39). This self-limiting nature of coccidial infections is widely known in chickens and other poultry. There is no stimulation of cross-protective immunity between species of coccidia. Thus, several outbreaks of coccidiosis are possible in the same flock, with different species involved in each. Breeder pullets and layer pullets are at greatest risk because they are kept on litter for 20 weeks or more. Normally, the infections with *E. acervulina, E. tenella*, *E. mitis, E. mivati, E. praecox*, and *E. maxima* are seen at 3–6 weeks of age and then *E. necatrix* at 8–18 weeks of age. *E. brunetti* is seen both early and late.

Coccidiosis rarely occurs in layers and breeders during the laying cycle, because of prior exposure to coccidia and resulting immunity. If a flock is not exposed to a particular species early in life or if immunity is depressed because of other diseases, outbreaks may occur after layers are moved to production houses. Outbreaks of any species in layers can reduce or eliminate egg production for several weeks.

Transmission and Vectors

Ingestion of viable sporulated oocysts is the only natural method of transmission. Infected chickens may shed oocysts in the feces for several days or weeks. The oocysts in feces become infective through the process of sporulation in about 2 days. Susceptible birds in the same flock may ingest the oocysts through litter-pecking or the contamination of food or water.

Although no natural intermediate hosts exist for the *Eimeria* spp., oocysts can be spread mechanically by many different animals, insects, contaminated equipment, wild birds, and dust. Oocysts generally are considered resistant to environmental extremes and to disinfectants, although survival time varies with conditions. Oocysts may survive for many week in soil, but survival in poultry litter is limited to a few days because of the heat and ammonia released by composting and the action of molds and bacteria. Viable oocysts have been reported from the dust inside and outside broiler houses, as well as from insects in poultry litter (39). The darkling beetle, common in broiler litter, is a mechanical carrier of oocysts. Transmission from one farm to an-

other is facilitated by movement of personnel and equipment between farms and by the migration of wild birds, which may mechanically spread the oocysts. New farms may remain free of coccidia for most of the first growout of chickens until the introduction of coccidia to a completely susceptible flock. Such outbreaks, often more severe than those experienced on older farms, are often called "the new-house syndrome."

Oocysts may survive for many weeks under optimal conditions but will be quickly killed by exposure to extreme temperatures or drying. Exposure to 55°C or freezing kills oocysts very quickly. Even 37°C kills oocysts when continued for 2–3 days. Sporozoites and sporocysts can be frozen in liquid nitrogen with appropriate cryopreservation technique, but oocysts cannot be adequately infiltrated with cryoprotectants to effect survival. Threat of coccidiosis is less during hot dry weather and greater in cooler damp weather.

Diagnosis of Coccidiosis

Coccidiosis can best be diagnosed from birds killed for immediate necropsy. Attempts to identify characteristic lesions in birds that have been dead for 1 hour or longer are frustrated by the postmortem changes in the intestinal mucosa. The entire intestinal tract should be examined. A microscope should be available for viewing endogenous forms on questionable lesions. The finding of a few oocysts by microscopic examination of smears from the intestine indicates the presence of infection, but not a diagnosis of clinical coccidiosis. Coccidia and mild lesions are present in the intestines of birds 3–6 weeks old in most flocks. Coccidiosis should be diagnosed if the gross lesions are serious or if other economic parameters are threatened. Diagnosis should be based on finding lesions and confirming microscopic stages on necropsy of typical birds from the flock, rather than from culls. Cryptosporidia may be found in chickens or turkeys but are distinguished easily from *Eimeria* by their small size and location in the brush border of the mucosal cells (14, 19).

Microscopic Examination

Developing schizonts, gametocytes, and oocysts of coccidia may be seen in smears taken from the suspected lesion. A small amount of mucosal scraping should be diluted with saline on a slide and then covered with a coverslip. Oocysts or macrogametes are most easily seen, but in many cases, the lesion is caused by maturing schizonts. Diagnostic characteristics which are of value include the clusters of the large schizonts of *E. necatrix* and *E. tenella,* the small round oocysts of *E. mitis,* or the large gametocytes of *E. maxima.* Presence of clusters of large schizonts in the midgut area is pathognomonic for *E. necatrix,* and a similar finding in the ceca indicates *E. tenella.* Oocysts associated with lesions in the duodenum are *E. acervulina, E. mivati,* or *E. praecox*, and oocysts associated with lesions in the lower gut are *E. mitis, E. mivati* or *E. brunetti*.

Oocyst size and shape are less useful as diagnostic characteristics in chickens than once thought, because of the extensive overlapping in size of the species. However, the combination of oocyst size, location in the gut, and appearance of the lesions gives considerable confidence in diagnosis. Measurement of 20–30 oocysts of the predominant type of oocyst usually gives a good indication of the size of the unknown species. This information is useful in conjunction with other observations in the identification of species in field cases.

Lesion Scoring

The severity of lesions is roughly proportional to the number of oocysts ingested by the bird and correlates with other parameters such as reduced weight gain, loss of skin pigmentation and diarrhea. The most commonly used practice is based on the system devised by Johnson and Reid (22). In this technique, a score of 0–4 is assigned to a bird where 0 = normal and 4 = most severe case. This technique is most useful in experimental infections, where the dose of oocysts and medicaments are controlled, and the species are known. In the field, lesion scoring is generally useful in gauging the severity of infections but may not correlate with microscopic scoring. Even though several species of coccidia may be present at some time, only 4 separate sections of the intestine are usually scored. These are 1) the duodenum (upper), with lesions of *E. acervulina, E. mivati*; 2) the midgut from the duodenum past the yolk sac diverticulum, with lesions of *E. maxima, E. praecox, E. necatrix,* and *E. mitis*; 3) the lower small intestine from the yolk sac diverticulum to the cecal junctures, with lesions of *E. mitis, E. necatrix, E. maxima and E. brunetti*; and 4) the ceca, where only *E. tenella* is found.

Microscopic scoring

As with lesion scores, the severity of coccidiosis can be judged by the number and appearance of parasite forms seen upon microscopic examination of smears from the mucosa, lumen or feces. Microscopic scoring is particularly useful for detecting and rating species that do not produce easily seen gross lesions, such as *E. mitis, E. hagani* and *E. praecox.*

Droppings Score

In laboratory infections, the droppings score may be used in the same manner as lesion score for a rapid and fairly reliable rating of the infection (31). The extent of abnormal droppings is rated on a scale of 0–4, where 4 = maximum diarrhea, with mucus, fluid, and/or blood. This technique has obvious complications where birds are infected with more than one species of *Eimeria.*

Histopathology Methods

Ordinary methods in histopathology are satisfactory for routine examination of tissues infected with coccidia. Staining of sections with H & E or other common histologic stains will demonstrate developing stages. Specialized techniques will identify specific stages: Staining with Schiff's reagent gives a brilliant red color with the polysaccharide associated with the refractile body and with wall-forming bodies in the macrogamete. Monoclonal antibodies conjugated with fluorescent markers such as fluorescein are very useful in research because specific stages of parts of cells can be readily identified.

Procedures Used in Species Identification

Most of the species of coccidia are easily identified by attention to well-established biological characteristics (Table 28.1,

28.3)(25, 30, 32, 43). The largest oocysts belong to *E. maxima,* making it easily distinguished from other species. Some species are identified easily by the location and appearance of gross lesions in concert with the size of oocysts or schizonts (*E. acervulina, E. maxima, E. necatrix, E. tenella*). The lesions produced by other species are not reliably distinct, and oocyst sizes overlap with those of other species. For *E. praecox,* the best method is determination of prepatent period by timed inoculation of birds in laboratory cages. Oocysts produced in less than 90 hours can only be *E. praecox*. *E. brunetti* oocysts are indistinguishable from those of *E. praecox*, *E. tenella,* and *E. necatrix* on size alone, but the location in the lower gut and appearance of the lesions are reliable indicators. *E. mitis* is located in the mid-lower gut, has small subspherical oocysts, and has a prepatent period of 99 hours, separating it from *E. brunetti*.

Because of the overlapping size, area parasitized, and lack of distinct lesions, it has been difficult to separate *E. hagani* from other species of the duodenum with small oocysts. In this case, immunization tests were very useful (36). Poultry develop immunity to reinfection after inoculation with *Eimeria*, but there is no cross-protection between species. This strict specificity of immunity has been exploited as a technique for distinguishing species of coccidia for taxonomic purposes. This test requires pure cultures of the test species and test animals reared in isolation for mono-immunization and challenge. When *E. hagani* oocysts were used to immunize chickens, the resulting immunity protected against reinfection by the same culture but not against other species. Conversely, birds immunized with other species were not protected against infection with the culture of *E. hagani*. Overall, the technique is time consuming, requires extensive laboratory isolation facilities, and access to pure cultures of known species of coccidia but may be useful as a research tool when used in concert with other tests or observations.

Preservation of Coccidia for Experimental Work

Droppings or litter collected in the field, or intestinal contents in the diagnostic lab, can be saved for isolation of coccidia in a solution of 2–4% potassium dichromate. Aeration of oocyst suspensions is necessary to allow sporulation. A good-quality aquarium pump is highly effective and can be regulated with valves and tubes to service several bottles at one time. For short-term storage, suspensions of oocysts may be refrigerated at temperatures above 4°C. Freezing temperatures quickly kill coccidial oocysts, as do elevated temperatures. Oocysts are quickly killed by storage at 37°C or higher.

Prevention and Control

Control of Coccidiosis by Chemotherapy

Early emphasis in chemotherapy was centered on the treatment of outbreaks with sulfonamides or other compounds as soon as signs of infection were apparent. The concept of preventive medication emerged with the realization that most of the damage is done by the time signs of coccidiosis are widespread in a flock. Today, almost all broiler flocks receive preventive medication (Table 28.2). Treatment is used as a last resort or when other programs have failed. The historical aspects of chemotherapy have been reviewed extensively by McDougald (27). Consult a current Feed Additives Compendium for up-to-date information on approved products (12).

Characteristics of Anticoccidial Drugs

All types of drugs used for coccidiosis control are unique in the mode of action, the way in which parasites are killed or arrested, and the effects of the drug on the growth and performance of the bird. Following are the most important characteristics.

Spectrum of Activity. There are several important species of coccidia in chickens, several more in turkeys, and many others in other hosts. A drug may be efficacious against one or several of these parasites; very few drugs are equally efficacious against all.

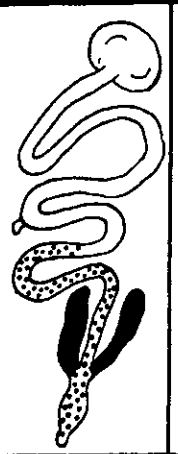
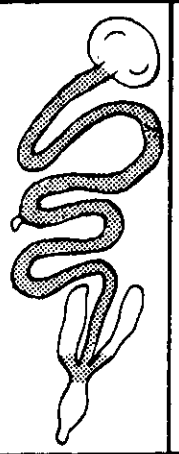
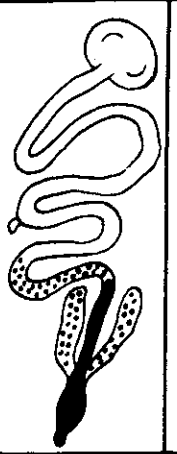
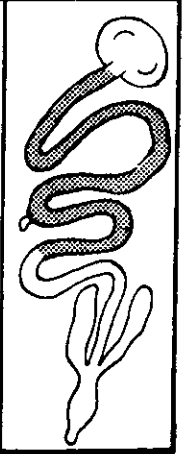
Mode of Action. Each class of chemical compound is unique in the type of action exerted on the parasite, and even in the developmental stage of the parasite most affected. The chemical mode of action of some drugs is known to be a highly detailed event, and the action of other drugs remains a mystery. The sulfonamides and related drugs compete for the incorporation of PABA and metabolism of folic acid. Amprolium competes for absorption of thiamine by the parasite. The quinoline coccidiostats and clopidol inhibit energy metabolism in the cytochrome system of the coccidia. The polyether ionophores upset the osmotic balance of the protozoan cell by altering the permeability of cell membranes for alkaline metal cations.

Endogenous Stage Affected. The coccidia are prone to attack by drugs at various stages in the development in the host. Totally unrelated drugs may attack the same stage of parasite. The quinolones and ionophores arrest or kill the sporozoite or early trophozoite. Nicarbazin, robenidine, and zoalene destroy the first- or second-generation schizonts, and the sulfonamides act on the developing schizonts and on the sexual stages. Diclazuril acts in early schizogony with *E. tenella* but is delayed to later schizogony with *E. acervulina* and to the maturing macrogamete with *E. maxima.* The time of action in the life cycle has been construed as having significance in the use of drugs in certain types of programs in which immunity is desired, but there is no good evidence that this is true under practical conditions.

Coccidiocidal versus Coccidiostatic. Some drugs kill the parasite, but others only arrest development. When coccidiostatic medication is withdrawn, arrested parasites may continue to develop and contaminate the environment with oocysts. In such cases, a relapse of coccidiosis is possible. In general, the coccidiocidal drugs have been more effective than those that are coccidiostatic.

Effects of Drugs on the Target Animal. Most compounds used in animal feeds have good "selective toxicity," providing toxicity for the parasite but being nontoxic to vertebrates. Unfortunately, toxicity and side effects of drugs on the host are possible where formulation errors lead to overdose. Sometimes, a drug may exhibit side effects at the recommended use level. Some of the toxicity may be the result of management, genetics, nutrition, or other in-

Table 28.3. Diagnostic characteristics of *Eimeria* in turkeys.

SPECIES → CHARACTERISTICS ↓	*E. adenoeides*	*E. dispersa*	*E. gallopavonis*	*E. innocua*	*E. meleagridis*	*E. meleagrimitis*	*E. subrotunda*
Lesions Occasional lesions Parasites no lesions Species distinctive							
Macroscopic lesions	liquid feces with mucus and flecks of blood, **loose whitish cecal cores**	cream-colored serosal surface, dilation of intestine, yellowish mucoid feces	edema, ulceration of mucosal ileum, yellow exudate, flecks of blood in feces	none	cream-colored ceca, formation of caseous plug, a few petechial hemorrhages	spotty congestion and petechiae from duodenum to ileum, dilation of jejunum, casts	none
Length × Width (in um) Length = Width =	Av = 25.6 × 16.6 18.9 - 31.3 12.6 - 20.9	Av = 26.1 × 21.0 21.8 - 31.1 17.7 - 23.9	Av = 27.1 × 17.2 22.7 - 32.7 15.2 - 19.4	Av = 22.4 × 20.9 18.57 - 25.86 17.34 - 24.54	Av = 24.4 × 18.1 20.3 - 30.8 15.4 - 20.6	**Av = 19.2 × 16.3** **15.8 - 26.9** **13.1 - 21.9**	Av = 21.8 × 19.8 16.48 - 26.42 14.21 - 24.44
Oocyst shape and index length/width	ellipsoidal **1.54**	broadly oval **1.24**	ellipsoidal **1.52**	subspherical **1.07**	ellipsoidal **1.34**	ovoid **1.17**	subspherical **1.10**
Minimum sporulation	24 hr	35 hr	**15 hr**	**under 45 hr**	24 hr	**18 hr**	**48 hr**
Prepatent period (minimum)	103 hr	**120 hr**	**105 hr**	**114 hr**	110 hr	103 hr	**95 hr**
Refractile body	yes	no	yes	no	yes	yes	no
Pathogenicity	+ + + +	+	+ + + +	none	**none**	+ + + +	none

teraction, and in other cases, the margin of safety is just too narrow. Environmental interaction is possible with nicarbazin, which interacts with high temperatures and high humidity to produce excess mortality. Also nicarbazin is highly toxic to layers, first causing a bleaching of brown-shelled eggs, mottling of yolks, reduced hatchability, and reduced production. The ionophores are highly toxic at elevated doses, causing a transient paralysis in mild overdoses or a permanent paralysis and mortality in more severe cases. Monensin was once thought to interact with methionine to reduce feather growth, but this relationship is not clear. Under some conditions, lasalocid will stimulate water consumption and excretion, resulting in a wet litter. With slight overdoses, most of the ionophores depress weight gain under laboratory conditions. A withdrawal period of 5–7 days is often practiced to allow "compensatory growth" to make up for the lost gain. The ionophores are known for their toxicity to other animals. Thus, monensin and salinomycin are highly toxic to horses. The lethal dose—50% (LD_{50}) for monensin in horses—is about 2 mg/kg body weight. Salinomycin is highly toxic to turkeys at levels greater than 15 g/ton and causes excessive mortality at the level recommended for use in chickens (60 g/ton), while monensin and lasalocid are well tolerated in turkeys at the level used for chickens.

Programs for Use of Anticoccidial Drugs in Broilers

In broilers, the objective is usually to produce the maximum growth and feed efficiency with minimum of disease, and in layers or breeders, the objective may be immunization. The choice of a product or program may depend on the season of the year or other factors which affect exposure. Several types of programs are practiced:

Continuous Use of a Single Drug. Often, a single product will be used from day 1 to slaughter, or with a withdrawal period of 3–7 days. Most products are approved for use until slaughter, but producers withdraw medication for economic or other reasons.

Shuttle or Dual Programs. The use of one product in the starter and another in the grower feed is called a "shuttle" program in the United States and a "dual" program in other countries. Some programs might contain as many as 3 drugs, with one drug in the starter, another in grower and yet another in the finisher. The shuttle program usually is intended to improve coccidiosis control. Intensive use of the polyether ionophore drugs for many years produced strains of coccidia in the field that have "reduced sensitivity" to the ionophores. It is a common practice to use another drug such as nicarbazin, diclazuril, or clopidol in either the starter or grower feed to bolster the anticoccidial control and take some pressure off of the ionophore. In other cases, the order of these drugs is reversed. The use of shuttle programs is thought to reduce buildup of drug resistance. At times, a high percentage of producers use some type of shuttle program.

Rotation of Products. It is considered sound management to make periodic changes in anticoccidial drug use. Most producers in the USA consider changes in the spring and in the fall. Rotation of drugs may improve productivity because of the buildup of isolates or species of coccidia that have reduced sensitivity after products have been used for a long time. Producers often notice a boost in productivity for a few months after a change of anticoccidial drugs. The seasonal rotation of products is intended to correspond with the intrinsic properties of the drugs. In the USA, nicarbazin must be used principally in the cooler months of the year, which also corresponds with maximum coccidiosis challenge. In the summer months, coccidiosis challenge tends to be milder, so weaker anticoccidials are used.

Drug Resistance

The development of tolerance to drugs by coccidia after exposure to medication is the most serious limitation to the effectiveness of these products. Surveys reveal widespread drug resistance in coccidia in the United States, South America, and Europe (16, 20, 23, 25, 31, 32). Even though coccidia develop less resistance to some drugs than to others, long-term exposure to any drug will produce a loss in sensitivity and, eventually, resistance. Drug resistance is a genetic phenomenon, and when established in a line of coccidia, will remain for many years or until selection pressure and genetic drift forces return to sensitivity in the population. Drugs such as the quinolones and clopidol have a well-defined mode of action, and resistance develops quickly as coccidia are selected with cytochromes, which do not bind as readily to the drug. The polyether ionophores, in contrast, have a more complicated mode of action involving the mechanisms of active transport of alkaline metal cations across cell membranes, and it has taken many years for coccidia to become tolerant, and in some cases, completely resistant. Many other drugs appear to be intermediate in selecting resistance in coccidia. The primary defense against drug resistance is the use of less intensive programs, shuttle programs, and frequent rotation of drugs. Rotation of programs, used alone, will not prevent the development of resistance. In some instances coccidia are able to become resistant to drugs after only a few months of use, and once developed, drug resistance is slow to dissipate. In recent years it has become a common practice to incorporate live coccidiosis vaccines in the rotation program, reasoning that the drug-sensitive vaccine strains tend to replace the drug resistant wild types. This approach has had demonstrable effects on the drug sensitivity profile on farms where it has been practiced.

Anticoccidial Drugs Used for Broilers in the United States

The products currently approved for use in chickens in the United States are listed in Table 28.2. Not all are still available commercially, but the approvals remain. Those used at present include monensin, narasin, salinomycin, semduramicin, and lasalocid (polyether ionophores), diclazuril, nicarbazin, amprolium 1 ethopabate, decoquinate, clopidol, sulfadimethoxine 1 ormetoprim, and sulfaquinoxaline. A product combining narasin with nicarbazin is also used, to take advantage of synergism between these molecules. Other products listed with approvals but lacking in significant activity include chlortetracycline and oxytetracycline. These products may prevent mortality from coccidiosis when given at high levels because of antibacterial activity but are

not of much value in general use. The polyether ionophores became the drugs of choice for prevention of coccidiosis in 1972 and remain the most extensively used today. Other drugs, such as clopidol, diclazuril, halofuginone, nicarbazin and robenidine, are used mostly in shuttle programs as an adjunct to the ionophores.

Immunization during Medication Programs in Broilers

Chickens develop immunity to coccidiosis after natural exposure and may even develop substantial immunity while receiving anticoccidial drugs (7, 18). The poultry industry has learned to take advantage of this phenomenon, practicing longer withdrawal programs of 2–3 weeks in some instances.

Coccidiosis Vaccines

Considerable research on coccidiosis vaccines in recent years has produced new live products. Increasingly, these products are finding use in the broiler industry. When live oocysts of coccidia are given to chickens at an early age, protection against the species contained in the inoculum is stimulated. The virulence of coccidia in these vaccines is attenuated largely by the size of the dose and by the means of administration. Some vaccines sold internationally or under development in the USA contain modified live coccidia with attenuation by genetic selection for short life cycle development. The use of coccidiosis vaccines in broilers has been limited by the possibility of adverse reactions, particularly a negative effect on feed efficiency. More recent advances in administration methods have overcome much of this limitation. The Coccivac products pioneered in this growing family, which now includes several other live vaccines in various countries (Coccivac®, Immucox®, Paracox®, Livacox®, BioVet®, Advent®, Nobilis®, In-OvoCox®, and others). Some new live vaccines have been prepared from attenuated lines of oocysts (e.g., Paracox7 and Livacox7). These vaccines normally contain 3 or more species of *Eimeria,* which are thought to be the most important. The *Eimeria* infecting poultry immunize only against themselves, so that the vaccine will only protect against the included species. As is well known, several species not normally included in the vaccines are capable of causing depressed gain, poor feed conversion, and loss of skin color, and are sometimes the cause of apparent vaccine failure. The success of some vaccines may depend more on a novel administration technique rather than attenuation. One experimental product was encapsulated in alginate beads and then mixed into the starter feed for "trickle administration." Other methods presently used are spray cabinet administration, direct eye-spray, *in -ovo* inoculation, or spraying the oocysts directly into feed or water in the poultry house. One product is mixed into gels, which are placed into the chick boxes for the chicks to eat (8). Other experimental approaches include inoculation of parasites or antigens *in ovo* and inoculation via the yolk sac diverticulum.

Monoclonal antibody technology has led to the identification of coccidial proteins, which offer some protection from infection when inoculated into young chicks. These proteins can be made in quantity if the gene that encodes the protein is cloned into a bacterial cell. Research is in progress identifying broad-spectrum antigens and appropriate routes of administration. One product based on this approach is CoxAbic®, which is composed of an antigen developed from a monoclonal protein produced in the gametocyte of *E. maxima*. The latter vaccine is given to hens in 2 doses, to confer maternal protection during the first 3 weeks of brooding.

Control Programs Used in Breeders and Layers

Pullets started on the floor and later reared as caged layers are not as dependent on immunity to coccidiosis as are floor layers. They are often protected against coccidiosis with preventive medication, as with broilers until they are moved to cages. Breeder pullets that will be kept on the floor during lay should have immunity to coccidiosis. Controlled exposure vaccination can be given by means of commercially produced live products (described above). Natural or "accidental" exposure assumes the presence of oocysts of important species. A broad-spectrum anticoccidial drug is sometimes given at the lowest approved level to provide protection for 6–12 weeks. Some producers reduce the level of the drug during the final 4 weeks in a step-down program, although as mentioned previously, chickens tend to develop immunizing infections despite the presence of the drug. This approach allows moderate numbers of coccidia to develop in the birds, stimulating the host immune system to protect against serious outbreaks. Such exposure rarely is insufficient to protect against all species, because not all species are present throughout the growing period. Outbreaks of *E. necatrix* have sometimes occurred at 8–16 weeks, after all medication has been stopped. Climatic and seasonal conditions may add to the inherent uncertainties of this method.

Disinfection and Sanitation

Older recommendations for coccidiosis control often suggest directions for sanitation and disinfection to prevent outbreaks. Most of these are no longer considered valid because 1) there have been too many failures in such programs; 2) oocysts are extremely resistant to common disinfectants; 3) complete house sterilization is never complete; and 4) an oocyst-sterile environment for floor-maintained birds could prevent early establishment of immunity and allow late outbreaks. In addition to disinfectants normally used in poultry houses, specific products have been used to target the oocyst for destruction. A product available in some countries contains an ammonium salt and sodium hydroxide (OO-cide®).

Chickens reared in cages rarely suffer outbreaks of coccidiosis. The exceptions are usually in single rows of cages in which there has been accidental fecal contamination of feed or water.

Coccidiosis in Turkeys

Coccidiosis in turkeys is common but is often unrecognized because the lesions in turkeys are less spectacular than those in chickens. Several species infect turkeys, but only about 4 are economically important. Typical signs of coccidiosis in turkeys are watery or mucoid diarrhea, blood-streaked feces, ruffled feathers, anorexia, and general signs of illness. Recovery is quick, so lesions could go undetected at necropsy. Several species have been found in commercial turkey farms throughout the United

States (8). Coccidia infecting domestic turkeys also infect wild turkeys. The common species of *Eimeria* found in commercial turkey operations are *E. meleagrimitis, E. adenoeides, E. meleagridis,* and *E. dispersa. E. gallopavonis* is seen in a low percentage of flocks. Range-rearing of turkeys can add significantly to the exposure of wildlife to coccidiosis and other diseases.

Turkeys of all ages are susceptible to primary infection, but birds older than 6–8 weeks are considered more resistant to the disease; they can suffer weight loss and morbidity but are not killed as easily as are younger birds. Reductions in the rate of weight gain are often unrecognized until adequate coccidiosis control measurers have been instituted.

Etiology

Seven species of *Eimeria* have been described in turkeys in the United States. Identifying characteristics of each species are listed in Table 28.3. *E. innocua and E. subrotunda* have been so rarely recovered that further work will be required to re-establish the validity of these species.

Besides the *Eimeria,* species reported from the turkey include *Isospora* and *Cryptosporidium* (see the next section). The *Eimeria* spp. are strictly intestinal, contrasting with *Cryptosporidium*, which may cause both respiratory and intestinal infection (17). The most pathogenic species of *Eimeria* are *E. adenoeides, E. meleagrimitis, E. gallopavonis*, and *E. dispersa.* Differentiation of oocysts of the pathogenic species from those of milder species is difficult because some of the species are poorly described. For instance, differentiation of *E. adenoeides* and *E. meleagridis* is difficult, as they inhabit the ceca and have oocysts that are fairly simlilar.

Eimeria adenoeides *Moore and Brown 1951*

Gross lesions appear primarily in the ceca but extend to the lower small intestine and cloaca. Cecal contents are often hardened into a core consisting of mucosal debris. The cecal and/or intestinal wall is often swollen and edematous. Oocysts are ellipsoidal and have a high shape index length/width (5/1.54). The oocysts average 25.6 × 16.6 mm. Typical oocysts of *E. adenoeides* are more pointed at one end than other species, aiding in recognition.

Pathogenesis

E. adenoeides is one of the most pathogenic of the turkey coccidia. Experimental infections of 25,000–100,000 oocysts in young poults may produce mortality up to 100% on day 5 or 6 PI. Turkeys several months old may lose considerable weight after infection. Outward signs of infection are apparent after 4 days PI. Feces are frequently fluid, may be blood-tinged, and may contain mucous casts. White or gray caseous cores may be produced in the ceca. In mild to moederate infections the cecal contents may be viscous and filled with oocysts. The lesions heal quickly, so no evidence of infection may be seen soon after the acute phase unless the cecal core remains.

Gross Lesions and Histopathology

By day 4 PI, the intestine may suffer congestion, edema, petechial hemorrhage, and mucous secretion. Five days PI, the ceca contain white caseous material, which condenses into a core. The serosal surface of the intestine appears pale and may be edematous and dilated.

Invasion of the submucosa by heterophils occurs throughout the intestine, especially in the lower small intestine, ceca and rectum. Epithelial cells at the tips of villi are most often invaded, but deep glands may also be parasitized. Edema is common deep in the muscular layers as the infection progresses. After day 5, regeneration of lost mucosa is rapid.

Eimeria dispersa *Tyzzer 1929*

The small intestine, principally the midgut region, is commonly parasitized, but some infection may occur in the cecal necks. Oocysts are large (average, 26.1 × 21.0 mm) and broadly ovoid (index = 1.24). Sporozoites lack a refractile body, and the oocyst wall is distinctively contoured and lacks the double wall common to other species. The prepatent period is 120 hours, longer than for other species.

Pathogenesis

Compared with some of the other species, the pathogenicity is low, but infection with $10^6 - 2 \times 10^6$ oocysts can cause reduction in rate of weight gain and diarrhea in young poults.

Natural and Experimental Hosts

The natural host of this species is apparently the bobwhite quail, in which the parasite is more pathogenic than in turkeys. This is the only *Eimeria* in chickens or turkeys known to infect more than one species. Experimental inoculation has produced patent infections in domestic and wild turkeys, Hungarian partridge (*Perdix perdix*), ruffed grouse (*Bonasa umbellus*), sharp-tailed grouse (*Pediocetes phasianellus campestris*), Japanese and bobwhite quail, and other pheasants. Infection in chickens often requires immunosuppression.

Gross Lesions and Histopathology

Three days PI, the duodenum appears cream-colored on the serosal surface. Later, the entire intestine may become dilated with thickening of the wall. Dilation continues on the fifth and sixth days, along with secretion of a cream-colored mucoid material containing denuded epithelium from the duodenum. Individual villi may become so dilated as to be visible to the naked eye.

The duodenum shows edema and progressively increasing congestion of capillaries. Separation of the epithelium and basement membranes may result in the lamina propria being exposed to a fibrin network or an open fluid-filled space. Necrosis is common on distal tips of villi. Parasites do not invade the glands.

Eimeria gallopavonis *Hawkins 1952*

Lesions are restricted to the area posterior to the yolk sac diverticulum and tend to be most severe in the lower small intestine and large intestine. Some foci of infection may be seen in the ceca. Oocysts are elongate, averaging 27.1 × 17.2 mm (index = 1.52). Differentiation of this species from *E. adenoeides* is often difficult. One difference is that *E. gallopavonis* has more rounded oocysts.

Pathogenesis

Experimental infection with $5 \times 10^4 - 2 \times 10^5$ oocysts causes mortality of 10–100% in 2–6-week-old poults. Mortality occurs 5–6 days PI.

Gross Lesions and Histopathology

On day 4 and 5 post-exposure, second- and third-generation schizonts are numerous in the ileum, necks of the ceca, and rectum. By day 6 the rectum is parasitized mostly with gamonts. Marked inflammatory and edematous changes on days 5–6 are followed by the sloughing of soft white caseous necrotic material containing numerous oocysts and flecks of blood on days 7 and 8.

Eimeria meleagridis *Tyzzer 1929*

Oocysts are ellipsoidal, averaging 24.4 × 18.12 mm (index 1.34). Visible lesions may be seen in the ceca with yellow-white caseous cores, but this species is considered virtually nonpathogenic. Oocysts resemble those of other pathogenic species in the ceca, and differentiation is difficult.

Pathogenesis

Most studies have characterized this species as almost nonpathogenic. Up to 5×10^6 oocysts produce little effect on the growth of 4–8-week-old poults. Earlier reports indicating greater pathogenicity may have come from mixed infections with *E. adenoeides.*

Gross Lesions and Histopathology

Nonadherent cream-colored caseous cecal cores are characteristics of infection in young poults. The core may be passed intact. The mucosa is somewhat thickened and may contain petechial hemorrhages in dilated portions of the ceca. The plugs disappear 5.5–6 days PI, and many oocysts may be found in cecal contents.

Edema and lymphocytic infiltration may be seen histologically, but less extensively than with *E. adenoeides* and *E. gallopavonis.* First-generation schizonts develop in the surface epithelium of the small intestine, but later stages occur in the cecal epithelium.

Eimeria meleagrimitis *Tyzzer 1929*

Infection with *E. meleagrimitis* is primarily upper intestinal but may spread throughout the small intestine in heavy infections. This is the most pathogenic of the upper-intestinal coccidia in turkeys. The oocysts are small (average, 19.2 × 16.3 mm) and ovoid (index = 1.17).

Pathogenesis

Experimental infection of young poults produces morbidity and mortality, lost weight gain, dehydration, and general unthriftiness. Inoculation of 2×10^5 oocysts produces some mortality and morbidity, but this species is not as pathogenic as *E. adenoeides.*

Gross Lesions and Histopathology

Infected birds show signs of dehydration. In the duodenum, enlargement and congestion are marked on days 5 and 6 of infection. Large amounts of mucus and fluid may be found in the lumen. Feces may contain occasional flecks of blood and mucous casts 5–7 days PI.

The tips of villi are most commonly parasitized, and the epithelium may be completely denuded, although hemorrhage is rare. Capillaries of the villi are markedly dilated and the tips edematous. Eosinophilic infiltration may begin as early as 2 hours PI and is extensive at the height of the infection.

Eimeria subrotunda *Moore, Brown, and Carter, 1954*

Poults inoculated with this species produced no gross lesions and it was considered nonpathogenic (34). Parasites develop primarily in the upper small intestine anterior to the yolk stalk diverticulum and are located in the epitehelial cells in the tips of the villi. Oocysts are subspherical (index = 1.099) and average 21.77 × 19.81 mm. Oocysts have no refractile granule.

Eimeria innocua *Moore and Brown 1952*

This species is said to produce no gross lesions and is considered nonpathogenic. The area parasitized is the small intestine, in the epithelial cells at the tips of villi. Oocysts are subspherical (index = 1.072), and average 22.4 × 20.9 mm. Oocysts have no polar granule. Prepatent period for oocyst production is 114 hrs.

Undescribed Species

Several species of coccidia that do not fit descriptions of established species have been isolated from wild or domestic turkeys but have not been adequately described or named. Thus, some difficulty may be expected in identifying coccidia found in field cases unless the pathology and appearance are distinctive.

Prevention and Control of Turkey Coccidiosis

Drugs effective in chickens are generally effective in turkeys, but the optimal level of application may vary, and the toxicity of some drugs is significantly higher in turkeys than in chickens.

Treatment

As in chickens, treatment of outbreaks in turkeys is less desirable than the prevention by chemotherapy or immunization. When treatment is necessary, application of amprolium (0.012–0.025% in water) or a sulfonamide (dosage depending on drug, often given 2 days on drug, 3 days off, and 2 days on, sometimes repeated a second week) is recommended. The toxicity of sulfonamides limits their usefulness for turkeys.

Control by Chemotherapy

Most producers use anticoccidial drugs continuously in the feed at least 8 weeks. Generally, poults are confined to a brooding facility at that time. Later, the birds may be moved to range or to other facilities. Drugs approved historically for use in feed include amprolium (0.0125–0.25%), butynorate (0.0275%), sulfaquinoxaline (0.0175%), sulfadimethoxine (0.006–0.25%) + ormetoprim (0.00375%), monensin (54–90 g/ton), halofuginone (1.5–3.0 ppm); diclazuril (1.0 ppm), and lasalocid (75–125 ppm). Not all are available.

Prevention with Planned Immunization

The principle of immunization by exposure to a small number of pathogenic oocysts of the important species of *Eimeria* was developed with chickens and is represented by a single product for turkeys in the United States (Coccivac-T7, Schering-Plough, Millsboro, Delaware) and in Canada (Immucox7, Vetech, Guelph, Ontario). The inoculum is sprayed on the feed during the first 1–7 days, or sprayed on the poults at one day of age at the hatchery, and causes a mild infection. There are risks inherent in use of virulent strains of coccidia, and occasional treatment at 3–4 weeks of age is necessary if one of the species multiplies too rapidly, but the program has been used with moderate success.

Coccidiosis in Geese

Numerous species of coccidia have been described from domestic and wild geese. The most prevalent and damaging in commercial flocks are *E. truncata,* which causes renal coccidiosis, and *E. anseris*, which causes intestinal coccidiosis. Renal coccidiosis may produce high mortality from the blockage of kidney function in young goslings. Coccidia may be introduced into domestic flocks by migrating and resident wild geese.

Eimeria truncata *Raillet and Lucet 1891*

Flock losses due to renal coccidiosis have been reported as high as 87% in Iowa. Geese aged 3–12 weeks are affected, although the disease is most acute in goslings. Signs of infection include depression, weakness, diarrhea with whitish feces, and anorexia. Eyes become dull and sunken, and wings are drooped. Survivors may show vertigo and torticollis. Birds quickly develop immunity to reinfection.

Oocysts and endogenous stages of *E. truncata* are found only in the kidneys or cloaca near the junction of the ureters. Diagnosis of *E. truncata* is ensured by finding the distinctive oocysts in the kidneys and ureters. Oocysts average 21.3 × 16.7 mm and have truncated ends.

Natural and Experimental Hosts

Although thorough cross-infection experiments have not been done in most cases, *E. truncata* has been reported from domestic and wild geese, ducks, and swans.

Gross Lesions and Histopathology

The kidneys may be enlarged and protrude from the sacral bed. The normal reddish brown is altered to light grayish yellow or grayish red. Pinhead-sized grayish white foci or hemorrhagic petechiae may be seen; they contain numerous oocysts and accumulations of urates. Invading and growing parasites may distort the kidney tubules to many times the normal size. Eosinophils and signs of necrosis are present in focal areas.

Eimeria anseris *Kotlan 1933*

The oocysts average 19.2 × 16.6 mm. Differentiation from the 14 species listed by Pellerdy (37) may be difficult.

Pathogenesis

E. anseris may produce anorexia, tottering gait, debility, diarrhea and morbidity, and sometimes mortality. The small intestine becomes enlarged and filled with thin reddish brown fluid. Catarrhal inflammatory lesions are most intense in middle and lower portions of the small intestine. There may be large whitish nodules or a fibrinous diphtheroid necrotic enteritis. Under dry pseudomembranous flakes, the oocysts and endogenous stages of the parasite are found in large numbers. Parasite stages invade epithelial cells of the posterior half of the intestine in closely packed rows. Developing gametocytes penetrate deeply into subepithelial tissues of the villi.

Treatment

Various sulfonamide drugs have been used in treatment of renal and intestinal coccidiosis of geese. Some studies indicated a favorable response, but, unfortunately, there have been no controlled experiments.

Coccidiosis in Ducks

Coccidiosis in ducks is sporadic but is of sufficient frequency to warrant more attention from researchers. Cases involving moderate to heavy mortality have been reported on domestic duck farms in New York, New Jersey, Hungary, and Japan. Coccidia were recovered from every farm sampled on Long Island, New York. Clinical and subclinical coccidiosis appears to be common and can produce morbidity and mortality as well as poor performance.

Species of Coccidia and Descriptions

Although 13 species of coccidia have been reported from domestic and wild ducks, the descriptions are often insufficient to use in diagnosis (32). Many species will remain in doubt until further work is completed. Coccidia in ducks may be of *Eimeria, Wenyonella,* or *Tyzzeria.* The genus can be determined readily from the sporulated oocyst. The oocysts of *Eimeria* have 4 sporocysts, each containing 2 sporozoites; *Wenyonella* have 4 sporocysts, each with 4 sporozoites; and *Tyzzeria* have 8 naked sporozoites not contained within sporocysts.

Tyzzeria perniciosa Allen 1936, from domestic ducks in the United States, have thin-walled oocysts measuring 10–12.3 × 9–10.8 mm and sporulate to produce 8 free sporozoites.

Wenyonella philiplevinei Leibovitz 1968 is the best described of the coccidia from ducks. It is found in the lower intestine from the posterior jejunal annular band to the cloaca. The prepatent period is 93 hours. The oocysts have three-layered walls, measure 15.5–21 × 12.5–16 mm (average, 18.7 × 14.4), have a micropyle at one end, 1–2 polar granules, and no oocyst residuum. Sporulation results in 4 sporocysts/oocyst, each containing 4 sporozoites.

Pathogenesis of Duck Coccidiosis

Signs of infection with *T. perniciosa* usually include anorexia, weight loss, weakness, distress, morbidity, and up to 70% mortality. Hemorrhagic areas are common in the anterior portion of the

intestine but may be found throughout. Bloody or cheesy exudate is common. The epithelial lining may be sloughed in long sheets. Parasite invasion may extend through the mucosal and submucosal layers as deep as the muscular layers. Acute hemorrhage as early as day 4 may be followed by death on days 5–6.

With *W. philiplevinei*, the effects are limited to 72–96 hours PI. Occasional petechial hemorrhages appear in the posterior ileal mucosa. Diffuse congestion is found in lower intestinal mucosa. In severe infections, mortality may occur on day 4.

Coccidiosis in Pigeons

Coccidiosis in pigeons is similar to, but less severe than, that caused in chickens by *E. necatrix.* Young pigeons suffer the greatest losses, but mortality may occur in birds as old as 3–4 mo.

The most frequently occurring species of coccidia in pigeons is *E. labbeana* (Labbe 1896) Pinto 1928. Oocysts are spherical or subspherical, averaging 19.1 × 17.4 mm.

Pathogenesis

Mortality of 15–70% has been reported in young pigeons in various parts of the world. Subclinical infections may persist in older birds for long periods. Immunity does not appear to be as self-limiting as reported for other species. Common signs of infection are anorexia, greenish diarrhea, marked dehydration, and emaciation. Droppings may be blood tinged, and the entire digestive tract may be inflamed. The common condition of going light is frequently attributed to coccidiosis.

Treatment

Favorable response has been reported after the use of sulfonamides in drinking water at the same or half the level recommended for chickens. A product was introduced in 1987 in France and Belgium for specific use in pigeons. The active ingredient is clazuril, a close relative of the diclazuril under development for use in chickens. This product is highly effective in treating coccidiosis in pigeons.

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Cryptosporidiosis

Larry R. McDougald

Introduction

Cryptosporidiosis is caused by small coccidian parasites of the genus *Cryptosporidium*, which live within the microvillous region of epithelial cells of the respiratory and gastrointestinal tracts of vertebrates. Naturally occurring infections have been reported from at least 9 different avian hosts. In chickens, turkeys, and quail, these parasites are primary pathogens that can produce respiratory and/or intestinal disease, resulting in morbidity and mortality. Species of *Cryptosporidium* infecting mammals have received considerable attention in recent years because of the widespread increase in immunocompromised hosts (5). Several reviews of the biology of *Cryptosporidium* are available (11, 12, 30, 42).

Human Health Importance

While cryptosporidiosis is important in humans and other animals, there is no evidence that *C. baileyi*, the avian species, causes any infection in other animals. Similarly, *C. parvum*, which is the predominant human pathogen, is not commonly seen in poultry. There is good evidence that *C. meleagridis,* an occasional but highly pathogenic species in turkeys, may actually be synonymous with *C. parvum.* Species reported from poultry are summarized in Table 28.4.

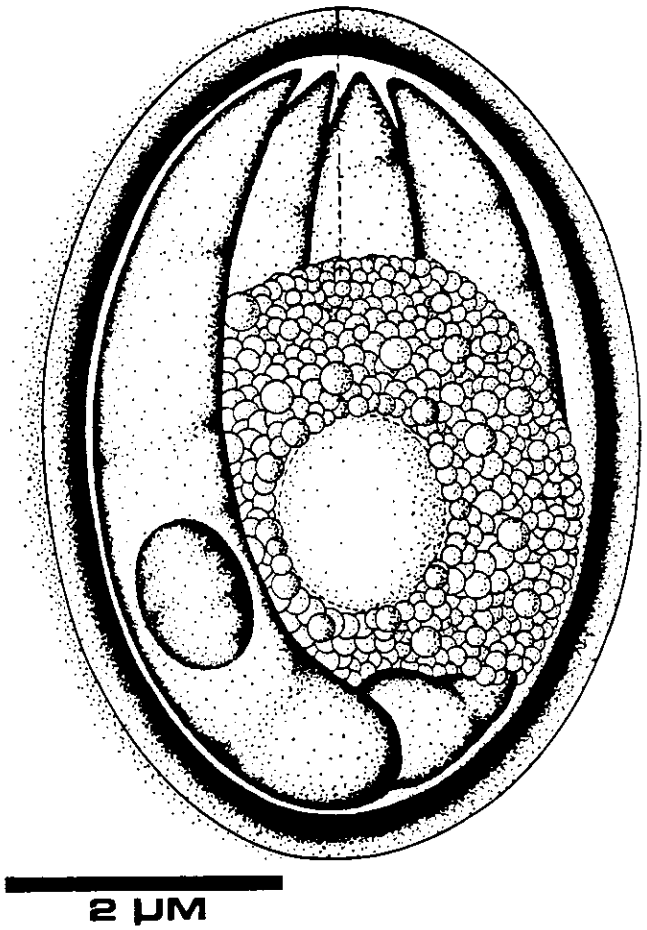
History and Taxonomy

The type species *C. muris* was described from laboratory mice by Tyzzer (39), who later also described many of the life cycle stages and named a second species, *C. parvum* (40, 41). Many other species were named from a variety of vertebrate hosts because researchers assumed an unwarranted degree of host specificity. Only a few are now considered valid. Two species (*C. baileyi* and *C. meleagridis*) infect chickens, turkeys, and quail (29). In chickens and turkeys, *C. baileyi* causes both intestinal (cloaca and bursa of Fabricius) and respiratory infections, and small intestinal infections of *C. meleagridis* infections are associated with diarrheal disease in turkeys and quail. An isolate that causes

Table 28.4. Distinguishing features of *Cryptosporidium* spp. infecting poultry.

Species	Host(s)	Site of Infection	Measurements of Oocysts (mm)
C. baileyi	Chicken, turkey, duck	Bursa of Fabricius, cloaca, respiratory epithelium	6.2 × 4.6 (mean), 6.3 – 5.6 × 4.8 – 4.5 (range)
C. meleagridis	Turkey, chicken	Small intestine	5.2 × 4.6 (mean), 6.0 – 5.6 × 4.8 – 4.5 (range)
Cryptosporidium spp.	Quail	Small intestine	Approximately 5

Source: See references 4, 22, 26.



28.5. Composite line drawing of an oocyst of *Cryptosporidium baileyi*. Note the 4 sporozoites surrounding the oocyst residuum and the suture in the two-layered oocyst wall. (8)

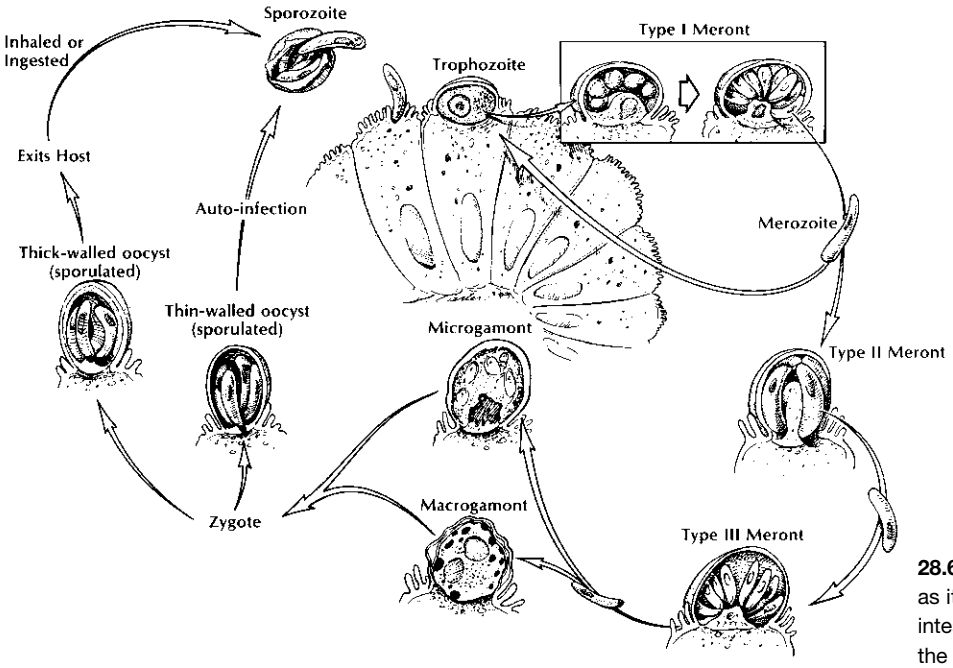
high mortality in quail, once thought distinct from *C. baileyi* and *C. meleagridis,* is now considered similar to *C. meleagridis* (29). However, as mentioned above, *C. meleagridis* may be a synonym of *C. parvum*.

Life Cycle and Morphology

Taxonomy of the coccidia is based on the differences in oocyst structure, sequence similarities in the 18s RNA gene, the heat shock gene (HSP-70), host specificity, and site of infection (4, 29, 41). In contrast to other coccidia found in poultry, *Cryptosporidium* spp. oocysts do not have sporocysts surrounding the sporozoites, 4 of which lie naked within the oocyst wall (Fig. 28.5). *C. baileyi* shows little host specificity among birds.

The life cycle of *Cryptosporidium,* like other true coccidia belonging to the suborder Eimeriorina, can be divided into 6 major developmental events (Fig. 28.6): excystation (release of infective sporozoites), merogony (asexual multiplication within epithelial cells), gametogony (formation of male and female gametes), fertilization (union of gametes), oocyst wall formation (to produce an environmentally resistant form), and sporogony (the formation of infective sporozoites within the oocyst wall).

The life cycle differs in several respects from that of *Eimeria* spp. infecting poultry (8). The intracellular stages of *Cryptosporidium* spp. are confined to the microvillous region of the host cell. Oocysts sporulate within the host cell and are infective when released in the feces. Oocysts are of 2 types: 1) thin-walled or 2) thick-walled. Thin-walled oocysts are not environmentally resistant and contain sporozoites surrounded by a single unit membrane. Upon release from the host cell, the sporozoites invade adjacent host cells. Thick-walled oocysts have a multilayered wall and are passed through the feces to infect other hosts. The majority of oocysts are the thick-walled form. In mammals, the thin-



28.6. Life cycle of *Cryptosporidium baileyi* as it occurs in the mucosal epithelium of the intestine (bursa of Fabricius and cloaca) and the respiratory tract of broiler chickens.

walled, autoinfective oocysts and type I meronts (asexual stages) cause reinfection within the same host, allowing severe infections to build up after ingestion of a small number of ingested oocysts. This is particularly important in immune-deficient hosts and may lead to a chronic life-threatening disease. Another feature of *Cryptosporidium* spp., which differs from *Eimeria* spp. in mammalian and avian hosts, is the frequent establishment of infections in the mucosal epithelium of a wide variety of organs. *C. baileyi* can infect the cloaca, the bursa of Fabricius, the upper and lower respiratory tracts, and the eyelids.

Diagnosis of cryptosporidiosis is difficult because of its diminuitive size and its location at the brush border of the epithelial cell. The tiny oocysts are difficult to see with light microscopy, as they are only a fraction of the size of other coccidian oocysts and have no features to make them stand out against a light background. For the same reason, they can be missed even in histopathology. Phase contrast and interference contrast microscopy are useful in wet preparations. Oocyst morphology may be useful for species identification (Table 28.4). Only *C. baileyi* can be identified on the basis of morphology alone because it is larger and more ovoid than *C. meleagridis* from turkeys or quail. *Cryptosporidium* isolated from quail will not infect chickens or turkeys. Thus, the species infecting quail can only be distinguished from *C. meleagridis* on the basis of host specificity. Oocyst walls of all forms are about 0.5 mm thick, colorless, and have no micropyle (Fig. 28.5).

Incidence and Distribution

Cryptosporidium spp. are prevalent in domesticated, caged, and wild birds, reported from 30 bird species. The reported worldwide distribution of *Cryptosporidium* spp. in avian hosts corresponds to the regions in which poultry health specialists and biologists have used appropriate diagnostic tools and will continue to expand as awareness of their importance as primary pathogens increases.

Cryptosporidiosis in Chickens

Cryptosporidium (probably *C. baileyi*) was diagnosed in 6.8% of 1000 consecutive histology cases of chickens in Georgia (16). In North Carolina, *Cryptosporidium* spp. oocysts were found in the feces of 9 (27.3%) of 33 broilers, 3 (10%) of 30 broiler breeders, and 1 (5.9%) of 17 layers (25). Using an ELISA, 22% of 454 broiler flocks in the Delmarva region were found to have birds that were seropositive for *Cryptosporidium* spp. when they were processed (6, 35). The number of positives among different companies sampled ranged from 2.8–40%. These investigations did not distinguish between intestinal and respiratory infections. Goodwin found respiratory cryptosporidiosis widespread in farms under contract to a broiler complex in North Georgia (16). The factors responsible for clinical expression of respiratory cryptosporidiosis are not understood but may cause high mortality and morbidity, with subsequent lower weight gains and higher feed/gain ratios (10). Experimentally induced respiratory and intestinal infections in broiler chickens have established the pathogenic potential of *C. baileyi* (2, 28). These and other data indicate that *Cryptosporidium* spp. are common in broiler chickens, and could have a significant impact on productivity and performance.

Pathogenesis and Epidemiology

Oocysts are picked up from heavy fecal contamination of the litter or cages. *C. baileyi* generally invades the epithelium of the cloaca and bursa of Fabricius. Respiratory infections apparently result from the inhalation or aspiration of oocysts that are present in the environment. As few as 100 oocysts can result in intestinal infections when given orally, or in respiratory infections when inoculated intratracheally. Oocysts of *C. baileyi* are infective at the time they are passed in the feces, and no vectors have been identified. Because *C. baileyi* can infect a variety of avian hosts, it is possible that wild birds may serve as carriers. Although *C. baileyi* is not infective for mammals, it is possible that rodents (mice and rats) and insects can serve as mechanical carriers (18).

Mild to heavy intestinal and respiratory signs can be seen as early as 3 days after inoculation of oocysts. Intestinal disease is usually mild. No overt signs of gastrointestinal disease occur in chickens receiving oocysts by gavage into the crop.

Signs of respiratory disease may appear within the first week after intratracheal (IT) inoculation of *C. baileyi* oocysts into 7- or 9-day-old broiler chickens, sometimes with severe morbidity and mortality (2, 9, 26). Oral inoculation of broilers with 4×10^5 oocysts produced only asymptomatic intestinal infections.

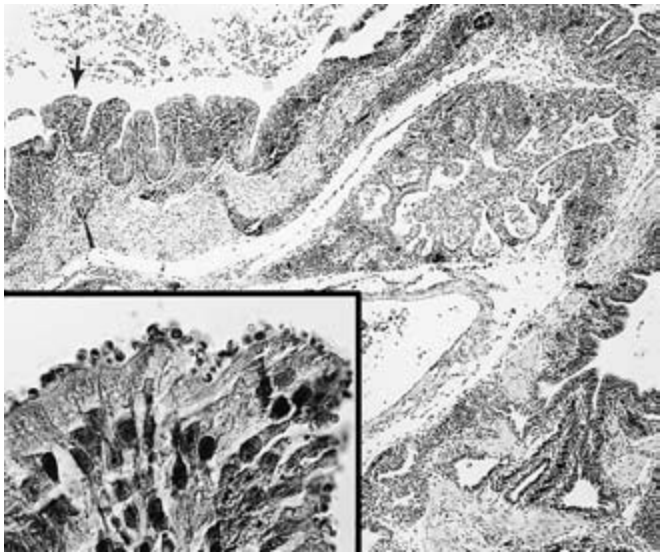
Respiratory signs of sneezing and coughing occur in most IT-inoculated chickens by 6 days postinoculation (PI). By 12 days PI, respiratory signs are more severe, and many of the birds extend their heads to facilitate breathing. Severe respiratory signs are present for about 3–4 weeks PI, after which there may be gradual improvement. Weight gains were depressed with respiratory infection but not with intestinal infections (9). Chickens were more resistant to intratracheal inoculation at 28 than at 7 or 14 days of age (28).

Airsacculitis and pneumonia can occur as early as 6 days but are more common 12–28 days following IT inoculation of *C. baileyi* oocysts. Early in the disease process, posterior thoracic air sacs are slightly thickened and contain foamy, clear to white or gray fluid. By day 12, air sacs may become very thick and contain white caseous exudate. The lungs of birds with severe airsacculitis are almost always affected and exhibit focal consolidation (10–80%), particularly in the ventral region. Abdominal air sacs may also be affected.

Histopathology of IT-inoculated chicks shows large numbers of parasites throughout the microvillous region of the epithelium lining the trachea and bronchi (15). Cilia are lost by replacement with developing parasites by 4 days PI (Fig. 28.7). By 12 days, almost all cilia may be replaced by developing parasites, and the mucociliary elevator function ceases in affected trachea and bronchi. Histologic lesions include epithelial cell hyperplasia, thickening of the mucosa by mononuclear cell infiltrates with some heterophils, loss of cilia, and discharge of mucocellular exudate into the airways. There is accumulation of mucus, sloughed epithelial cells, lymphocytes, macrophages, and parasites in the



28.7. The mucosal surface of the primary bronchi obtained from a broiler chicken 4 days after intratracheal inoculation of *Cryptosporidium baileyi*, as shown by scanning electron microscopy. Some developmental stages of the parasite can be seen among the cilia of the respiratory surface. At this stage of infection, the mucociliary elevator is probably still functional, and the bird would not have overt signs of respiratory distress. On days 10–18 after intratracheal inoculation, developmental stages of the parasite form a virtual monolayer on the respiratory surface. Few or no cilia can be found. (White)



28.8. *Cryptosporidium baileyi* in the lungs of a broiler chicken: Accumulation of lymphoid cells around bronchi 6 days after intratracheal inoculation of *C. baileyi* oocysts. (H & E stained histologic section) Inset: A higher magnification of the villus (arrow) showing the numerous developmental stages of the parasite on the epithelial surface.

tertiary bronchi and atria of the lungs. Affected lobules are expanded by accumulation of exudate and infiltration of mononuclear cells (Fig. 28.8). Affected air sacs lined with respiratory epithelium also contain large numbers of parasites and suffer similar changes.

Intestinal (cloaca and bursa of Fabricius) cryptosporidiosis in chickens (produced by *C. baileyi*) may result in histologic lesions but does not usually result in gross lesions or in overt signs of disease. Several reports suggest, however, that performance of broilers can be adversely affected. An unusually high mortality was associated with *C. baileyi* infection in the bursa of Fabricius, and there were lower pigmentation scores when inoculated birds were compared with noninfected controls (2, 19).

Interaction of *C. baileyi* and other respiratory pathogens predisposes birds to secondary invasion by *Escherichia coli* because of the disruption of the mucociliary elevator (9). Infectious bronchitis virus and *E. coli* also enhance the severity of *C. baileyi*-induced respiratory disease in chickens.

Cryptosporidiosis in Turkeys

Two species of *Cryptosporidium* found in turkeys are *C. meleagridis* (35) and *C. baileyi.* However, the description of *C. meleagridis* is indistinguishable from *C. parvum.* The intestinal (bursa of Fabricius and cloaca) and respiratory infections produced by *C. baileyi* are similar to those described previously for chickens (8, 9, 27).

Slavin reported small intestinal cryptosporidiosis (34) due to *C. meleagridis* in a flock of 10–14-day-old turkey poults. Illness was associated with diarrhea, unthriftiness, and moderate mortality. More than 30 years later, several outbreaks of this disease were reported (17, 43), although the number of reported cases is low.

Turkey poults infected with *C. meleagridis* may develop severe diarrhea. Numerous parasites are seen lining the brush border of the mucosa of the middle and lower small intestine. The gut becomes pale and distended with cloudy mucoid fluid and gas bubbles. Villi in the affected regions become atrophic; crypts become hypertrophic; and large numbers of lymphocytes, heterophils, and some macrophages and plasma cells accumulate within the lamina propria (17).

There are several case reports of severe respiratory cryptosporidiosis in commercial turkeys caused by *Cryptosporidium* spp. (probably *C. baileyi*) (14, 22, 32, 37). The disease may have upper or lower respiratory involvement. Upper respiratory infections may cause acute bilateral swelling of infraorbital sinuses, similar to that reported for birds infected with *Mycoplasma* spp., and serous conjunctivitis (14, 22). Case reports of lower respiratory tract infections reported signs including rattling, coughing, sneezing, and gasping (32, 37). The trachea and bronchi were colonized, with concomitant airsacculitis and pneumonia.

Microscopic lesions of the infected tissues included deciliation of the epithelium and inflammation.

Intratrachael (IT) inoculation with *C. baileyi* from the intestinal tract of broiler chickens into the trachea of turkeys produced respiratory signs similar to those observed in natural outbreaks (27).

Although there are reports of clinical outbreaks, the importance of *Cryptosporidium* spp. in commercially reared turkeys is not clear.

Cryptosporidiosis in Quail

Both respiratory and intestinal cryptosporidiosis have been reported in commercially grown quail, but the species involved has not been adequately described. Field reports suggest similiar respiratory disease and low mortality similar to that seen with cryptosporidiosis in chickens (38). Histologic examination revealed parasites in the microvillous region of epithelial cells lining the nasal cavity, trachea, bronchi, salivary glands of the roof of the mouth, esophageal glands, and bursa of Fabricius. Pathologic changes in the respiratory mucosa were similar to those described previously for chickens infected experimentally with *C. baileyi.* In another spectacular case of cryptosporidiosis, 5 successive hatches of 25,000 young quail (*Colinus virginianus*) developed severe, fatal intestinal cryptosporidiosis (23). Diarrhea developed 4–6 days after hatching, and mortality soon exceeded 90%. At necropsy, numerous developmental stages of the parasite were observed in the microvillous border of the small intestine. No parasites were observed in the cecum, colon, bursa of Fabricius, respiratory tract, or other tissues. Oocysts, obtained from the intestines of these infected quail, were not infective to day-old broilers. Based on recent work, this isolate was probably *E. meleagridis* (29).

A similar outbreak was reported from young quail due to a combination of *Cryptosporidium* sp. and a reovirus isolated from intestinal contents (33). Subsequent laboratory studies (20) suggested that the *Cryptosporidium* and not the reovirus was responsible for the intestinal disease.

Prevention and Control

There are no effective anti-cryptosporidial drugs or vaccines, and other approaches to the control are still experimental. Sanitation or disinfection may provide some help, but there are no proven programs that can be recommended.

Sanitation

The oocysts of *Cryptosporidium* spp. infecting poultry are remarkably resistant to chemical agents that readily kill most viral, bacterial, and fungal pathogens. Destruction of oocysts in commercial production facilities is not considered practical. In the laboratory, oocysts remain viable for months when stored at 4°C in a solution of 2.5% potassium dichromate. Oocyst viability is also maintained after a 10–15 minute incubation in 25% commercial bleach (sodium hypochlorite). Incubation of *C. baileyi* oocysts for 30 minutes at room temperature in each of 9 commonly used disinfectants mixed with water at the highest concentration recommended by the manufacturers had little or no effect on viability (36). Incubation in 50% ammonia resulted in the greatest reduction in excystation, and 50% commercial bleach destroyed many of the oocysts. Steam cleaning is a safe and effective means of disinfecting contaminated laboratory cages because oocysts are destroyed by temperatures greater than 65°C.

Immunity

A single intestinal and/or respiratory infection with *C. baileyi* can stimulate an immune response in broiler chickens of sufficient magnitude to clear the parasite from the infected mucosae and to protect the host against reinfection of the same species (6, 9). Experience with cryptosporidiosis in other animals suggests that immune protection may be short-lived. Oral or IT inoculation of oocysts into 8–14-day-old broilers results in heavy infections of the exposed mucosae for 14–16 days and then a rapid clearance of the parasite. High titers of circulating antibodies specific to *C. baileyi* can be detected after primary infections, and the birds exhibit a delayed hypersensitivity reaction to *C. baileyi* oocyst antigens. Data from laboratory studies and from a serologic survey suggest that acquired immunity may be important in the protection of broilers from cryptosporidiosis during the last several weeks of growout. Studies are needed to identify antigens of *Cryptosporidium* spp. that may be candidates for use as vaccines.

Diagnosis and Culture

Active infections in poultry, both respiratory and intestinal, can be diagnosed by identifying oocysts from fluids obtained from the respiratory tract or from the feces. Identification of *Cryptosporidium* spp. oocysts differs somewhat from techniques used for the oocysts of *Eimeria* spp. For viewing, oocysts are concentrated and observed by standard brightfield or phase contrast microscopy (7), acid-fast staining (13, 31), negative staining (4, 21), and staining with auramine-O for examination by fluorescence microscopy (25). These techniques allow one to readily distinguish *Cryptosporidium* spp. oocysts from yeast cells or *Blastocystis* that are often present in specimens.

Fecal or respiratory specimens can be collected and submitted fresh, in 10% formalin or in an aqueous solution of 2.5% potassium dichromate. A highly effective method of obtaining specimens in the field and in the laboratory is with moist cotton tipped swabs. Vigorous swabbing of the tracheal or cloacal epithelium will remove oocysts from the microvillous border. The swabs are placed in a tube containing 1 mL of water or fixative for transportation to the laboratory. *Cryptosporidium* infection can also be detected by demonstrating other stages of the life cycle from fresh or stained mucosal scrapings from the mucosa (24). Abbassi (1) described a semiquantitative microscopic slide flotation method that was reliable for *C. baileyi* in feces and organs of chickens. These parasites also appear in histologic sections stained with hematoxylin and eosin as 2–6-mm basophilic bodies within the brush border of the epithelial cells. Because of the small size of these parasites, transmission electron microscopy (TEM) is useful to reveal developmental stages and oocysts

within the host cells. Inoculation of chicken embryos (10 day) with oocysts of *C. baileyi* is a good method for propagation of this species in the laboratory, providing about 50% of the number of oocysts obtained from chickens (44).

Previous exposure to the parasite can be demonstrated by testing for serum antibodies specific to *Cryptosporidium* sp. by ELISA or other immunologic tests (6, 35).

Amplification of DNA sequences with PCR is a useful tool for identifying some species of *Cryptosporidium* (29), but studies with 8 DNA loci revealed homologies between *C. meleagridis* and the human pathogen *C. parvum*, which could not be resolved by PCR (3).

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Cochlosoma anatis Infection

Alex J. Bermudez

Introduction

The generic name *Cochlosoma* was created by Kotlan in 1923 to include a peculiar flagellate, *Cochlosoma anatis*, found in the intestines of the European domestic duck (25). The most striking feature of these flagellated protozoa is a distinct ventral adhesive disk. The significance of these enteric protozoa has been uncertain for many years. Previous editions of *Diseases of Poultry* suggest that *C. anatis* is either a parasite of unknown significance (15) or apparently nonpathogenic (17). These conclusions were based on the limited and conflicting published reports that were available. Recent case reports and research suggest *C. anatis* is a significant pathogen in both turkeys and ducks (1, 4, 7, 24). The parasite appears to cause limited pathology to the intestinal tract but causes a diarrhea and stunting of both turkey poults and ducklings. Research on this protozoon continues to be hampered by the fact that it cannot be cultured in artificial media (14) and must be collected from the feces or intestines of infected birds. These experiments cannot completely exclude bacteria, viruses, or other protozoa that may interact synergistically with *C. anatis*.

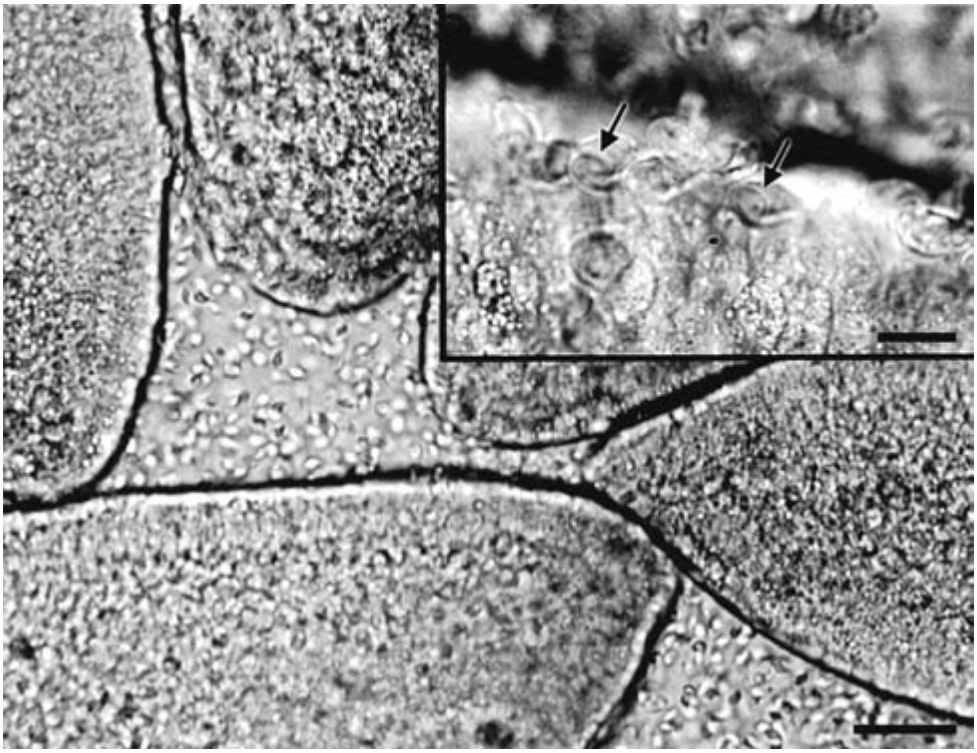
Etiology and Classification

Kotlan created the generic name *Cochlosoma* in 1923 to describe a protozoon identified in the duck (25). Tyzzer, in 1930, described 2 similar genera from the intestines of the ruffed grouse. He erected a new family, Cochlosomidae, to include the type genus *Cochlosoma* Kotlan (25), and *C. anatis* is the type species for this family (22). Numerous authors have noted similarities between *Cochlosoma* and both *Giardia* and *Trichomonas* (7, 11, 19, 21). The resulting question is whether *Cochlosoma* should be assigned to the order Retortamonadida or Trichomonadida. The adhesive disc is the primary similarity between *Cochlosoma* and *Giardia* (12). *Cochlosoma* has a parabasal apparatus, tubular axostyle, and crescent shaped pelta, all of which are similar to trichomonads (12). Pecka *et al.* conducted an extensive ultrastructural study of *Cochlosoma* in an attempt to more accurately classify these protozoa (22). In this report, a close relationship between *Cochlosoma* and Retortamonadida was excluded and the ultrastructural homology of C*ochlosoma anatis* with *Trichomonas* was demonstrated, justifying the classification of this genus within the order Trichomonadida Kirby, 1947 and family Cochlosomatidae Tyzzer, 1930, emend. (13, 22). These taxanomic relationships have been confirmed by the phylogenetic analysis of the small-subunit rRNA gene (10).

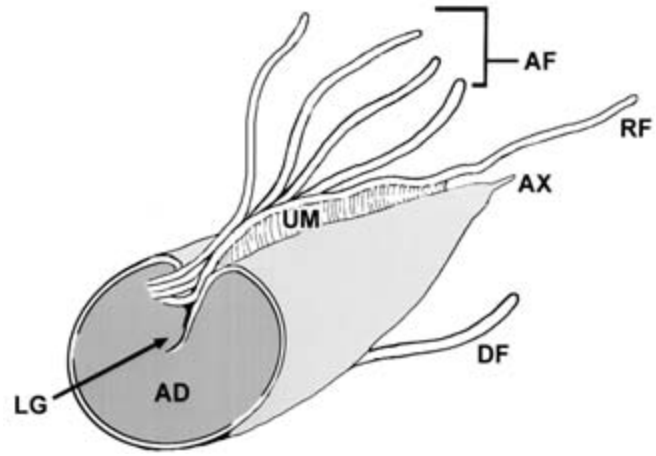
Morphology

By light microscopy, *C. anatis* is general pyriform to ovoid in shape, 6–12 microns long and 4–7 microns wide. The anterior end is wide and tapering to a narrowly rounded posterior end. A characteristic adhesive disc is on the anteroventral surface, which opens on the left side and is the predominate morphologic feature on light microscopy (Fig. 28.9). The parasite has a single nucleus, which can be distinguished readily on Giemsa or trichrome-stained impression smears (7). The trophozoites are distinguished easily from other protozoan flagellates in wet mount preparations by their characteristic motion. Flagella produce a whipping motion, which causes a stiff jumping or jerking motion and results in the parasite rotating around its long axis, as it moves forward. This motion is in contrast to the jerking motion of *Trichomonas* with its distinct undulating membrane and the rapid darting motion of *Hexamita* (19). Although *C. anatis* does possess a distinct undulating membrane based on scanning electron microscopy studies, this feature is not distinct on wet mount preparations of the parasite examined under standard light microscopy.

Numerous authors have described the morphology of *C. anatis* with scanning electron microscopy as is illustrated by Figure 28.10 (7, 14, 22, 27). On scanning electron microscopy, the trophozoite has a conical shape. The prominent ventral adhesive disc forms the base of the cone, and the body tapers toward the proximal end (7). The parasite has a prominent lateral grove, an undulating membrane, 6 flagella, and an axostyle (14). On the



28.9. *Cochlosoma anatis* as viewed by light microscopy in a wet mount preparation of mucosa of the jejunum of a turkey. Numerous protozoan cells are evident between the intestinal villi. Bar = 80 mm. Inset: Higher magnification; distinct ventral disc indicated by arrows. Bar = 10 mm.



28.10. *Cochlosoma anatis*: Prominent external features, ventral view. Ventral adhesive disc (AD), recurrent flagellum (RF), undulating membrane (UM), anterior flagella (AF), axostyle (AX), and anterior opening to the lateral groove (LG). The lateral groove runs the full length of the trophozoite adjacent to the undulating membrane. Not visible is the origin of the single dorsal flagellum (DF).

left side, the disc is interrupted by a lateral groove, which extends along the length of the body (22). Four anterior flagella emerge as 2 pairs, just above the lateral groove, on the left wall of the ventral disc (22, 27). These flagella tend to turn backward along the lateral groove being confined to the groove by their apical portions (22). The recurrent flagellum arises with the 4 anterior flagella and is associated with the undulating membrane and continues beyond it by a free trailing portion (22). The sixth flagellum arises to the left of the body midline on the dorsal surface (27). The distance of this flagellum from the anterior margin is about half the diameter of the ventral disk (27). The axostyle extends from the posterior end of the trophozoite as a thin projection (22). The organelle structure of *C. anatis* has been extensively characterized by Pecka *et al.* using transmission electron microscopy (22).

Transmission, Incubation Period, and Life Cycle

Bollinger and Barker reported that oral or cloacal inoculation of fecal material containing *C. anatis* reproduced the disease in ducklings (1). Ducks shed trophozoites by 7 days post inoculation (1). Oral transmission was also demonstrated experimentally in turkeys (14). Some turkeys were positive for *C. anatis,* based on intestinal mucosal scrapings, by 4 days postinoculation, and all poults were infected by 6 days post inoculation (14). Transmission was also readily successful when naïve turkeys were placed with infected turkeys. House flies have been implicated as a potential means of transmission from the environment to a susceptible host (18).

Reproduction in this species was recorded briefly by Kotlan. He observed longitudinal division and cysts with 4 or more nuclei but published no drawings of divisional stages or of the cysts and gave no dimensions for the cysts (22). Division of *C. anatis* by longitudinal fission has also been described in subsequent studies, but no cysts have been reported (11, 25). Studies by Evans *et al.* (8) document both the longitudinal binary fission of

C. anatis as well as pseudocyst formation. Pseudocysts also produced active *C. anatis* infection in turkeys (8).

Pathogenesis and Epidemiology

Natural and Experimental Hosts

Naturally occurring *C. anatis* infections have been described in the turkey, duck, goose, and coot (1, 7, 21, 25). Experimental *C. anatis* infections have been produced in bobwhite quail and chickens (14). *Cochlosoma* spp. infections have been reported in a wide variety of wild birds including the Eastern robin, American magpie, woodcock, waxbill, and a variety of finches (21, 23, 25). Only 2 *Cochlosoma* species have been recorded in mammals: *Cochlosoma pipistrelli* in bats of the family Phyllostomatidae (21) and *Cochlosoma soricis* in shrews (27). These reports suggest wild birds, particularly waterfowl, may serve as a reservoir for *C. anatis* and that mammals are probably not a reservoir for this protozoan.

Site of Infection

Cochlosoma is found in the cecum of the European domestic duck, and mainly in the colon or lower ileum of ducks (11). Travis found these protozoa to be common in the cloaca of ducks, frequently present in the colon and occasionally present in the cecum (25). Similarly, Watkins *et al.* found *C. anatis* to be present only in the last 10 cm of the colon and in the cloaca of ducks (27). One-day-old ducklings experimentally inoculated with *C. anatis* were infected in both the jejunum and colon 25 days post inoculation (1). In a goose, *C. anatis* was found primarily in the colon and also in the cecum (21).

In a study of infectious catarrhal enteritis (hexamitiasis) in turkeys, numerous poults were concurrently infected with *Cochlosoma* throughout the intestinal tract, and in adults, infection was noted in the region of the cecal tonsil (19). Cooper *et al.* describe a natural outbreak of enteritis in turkeys in which large numbers of *C. anatis* were found in the small intestines with isolated protozoa found in the ileum and none in the cecum or colon (7). In an experimental infection of turkeys, trophozoites were present in the duodenum, jejunum, and ileum of all birds and also the cecum and colon of some birds (14). *C. anatis* was present in the ileum of bobwhite quail and the cecum of one experimentally inoculated chicken (14).

Clinical Signs and Pathogenicity

Kotlan noted in his first description of *C. anatis* infection in ducks that the intestinal wall was swollen and catarrhal at the point where a mass of the flagellates were attached and the intestinal contents were mixed with blood. Kimura was unable to attach any pathogenic significance to this organism in ducks but noted inflamed intestinal tracts that were ascribed to a bacterial infection rather than to *C. anatis* (11). Travis did not observe pathogenic changes in the birds he studied (25). Severe runting and mortality were reported in ducklings that were infected with *C. anatis* (1). Numerous *C. anatis* with low numbers of *Trichomonas* and *Hexamita* were observed, and no other microscopic lesions were noted. *C. anatis* was highly suspected as the cause of the runting and the pathogenicity of *C. anatis* was then studied experimentally in ducks. Although the inoculum contained low numbers of *Hexamita* spp. and trichomonads and *Campylobacter jejuni* was isolated, it was concluded that *C. anatis* was at least implicated in the severe runting (reduced body weight and delayed feather growth) of ducklings. Many infected ducklings also died of secondary gram-negative bacterial septicemia (1). The same authors in a separate experiment found *C. anatis* infection increased intestinal villus length and altered mucosal enzyme concentrations in ducklings (2).

McNeil and Hinshaw found that *C. anatis* was often present in the intestines of turkeys in cases of hexamitiasis (19). They questioned the significance of this parasite in turkey poults, because in their experience, *C. anatis* was always found in association with Spironucleus (*Hexamita*) or in combinations with *Spironucleus* and *Salmonella*. Campbell reported a case of *C. anatis* infection in turkeys in which affected turkeys, 2–10 weeks of age, had severe catarrhal enteritis (6). Although the clinical signs in these cases were attributed to *C. anatis* because it was the predominate organism, *Trichomonas* and *Spironucleus* were also usually present. Cooper *et al.* identified *C. anatis* as the likely etiologic agent in a series of cases of diarrhea and enteritis in turkeys (7). Affected turkeys were depressed and ruffled, and many had a yellowish diarrhea. In this series of 6 cases, *C. anatis* was consistently present, although other potential viral or bacterial pathogens were also identified in individual cases. Affected turkeys were 7–12 weeks of age, and body weight gains were depressed an average of 16% at processing. During necropsy, small intestines were distended with fluid and ingesta. Dilated congested regions, focal intestinal distension, or bullae, were found in the duodenum and jejunum. Significant microscopic lesions were also noted in infected turkeys including blunting and fusion of the villi, a mixed cellular infiltrate of the lamina propria, and crypt hyperplasia. Because other enteric pathogens, including enteric viruses, were identified in some of these cases, it is possible that these microscopic changes were not entirely the result of a *C. anatis* infection. Recent experimental *C. anatis* infections in turkeys would suggest that this protozoan causes little or no microscopic change in the intestinal tract (3).

Experimental studies in turkeys were conducted that compared uninoculated controls, *C. anatis* infected poults, and poults inoculated with *C. anatis* and treated with metronidazole. *C. anatis* infection caused a significant decrease in weight gain and an increase in feed conversion in comparison to uninoculated controls or inoculated poults concurrently treated with metronidazole (5). An experimental study using turkey poults showed that a combination of *C. anatis* and turkey coronavirus was more pathogenic than either agent alone (24). However, both the *C. anatis* and coronavirus infected poults gained less than the negative controls. These findings are interesting in that *C. anatis* infections in turkeys often occur in combination with a variety of enteric viruses or other enteric pathogens. All these experimental findings with *C. anatis* are in general agreement with the case report by Cooper *et al.* in which a 16% decrease in body weight was noted in *C. anatis* infected flocks (7).

Finch aviaries have reported significant mortality in young

finches, 6–12 weeks of age, as the result of *Cochlosoma* infections (16, 23). Clinical signs included debility, dehydration, and the passing of whole seeds in the droppings.

The exact role of the ventral adhesive disc in the pathogenicity of *Cochlosoma* is still unknown. In multiple scanning and transmission electron microscopy images, a clear indentation in the mucosal brush border of the hosts with the same size and shape as the ventral adhesive disk suggests that the ventral disc serves for the attachment to the intestinal mucosa (7, 22, 27). It appears likely that this attachment to the microvillous border plays some role in the decreased production parameters seen in *C. anatis* infected turkeys and ducklings. Whether this attachment causes a direct pathologic effect, a mechanical blockage of nutrient absorption, or in some other way interferes with nutrient utilization has not been fully characterized.

Prevention and Control

Treatment

Multiple reports have shown that natural or experimental *Cochlosoma* infections can be treated successfully with members of the nitroimidazole family. However, these products are no longer available for use in poultry. Experimental infection of ducklings treated orally at 12 days post inoculation with 7.5 mg of metronidazole per 100g of body weight for 5 days resulted in complete compensatory weight gain (1). In an outbreak of *Cochlosoma* infection in waxbills, 250 mg/L of metronidazole in the drinking water for 3 days successfully treated the infection (23). During a survey for *Cochlosoma* infection in finches, the evaluation of the efficacy of metronidazole and ronidazole showed that at all dosages and duration of treatments investigated resulted in the clearance of the infection (9). Metronidazole and dimetridazole at 15 mg/L in the drinking water of turkeys did not reduce the number of organisms, but when given at either 30 or 60 mg/L, metronidazole, dimetridazole, and ronidazole successfully eliminated the organism (20). Metronidazole administered at 100 mg/L in the drinking water completely eliminated the negative production effects of *C. anatis* infection in turkeys (5).

Roxarsone at 0.002% in the drinking water did not reduce the number of *C. anatis* trophozoites in infected birds (20). The use of roxarsone in the drinking water at the recommended concentration (0.002%) improved the production performance compared with a *C. anatis* infected group but did not cure the *C. anatis* infection (3). Finally, roxarsone in the drinking water given at 2 times the label recommended dose (0.004%) for 2 days followed by 3 days at 0.002% prevented the negative production effects of a *C. anatis* infection and significantly decreased the parasitic burden (3). These results suggest roxarsone can be used as an effective treatment for *C. anatis* infection in turkeys, and this drug is the only effective treatment that can be used legally in food animals in the United States.

Prevention

As with most poultry diseases, the prevention of *C. anatis* infection can best be accomplished by not introducing the organism onto the poultry farm. Introduction by both human traffic and wild birds, particularly waterfowl, would appear to be the greatest risk factors for the introduction of this parasite onto the farm. *C. anatis* is killed in infected droppings that are allowed to dry at room temperature for 24 hours (3). Phenolic or quaternary ammonium disinfectants and 10% formalin all kill the protozoa with a 10-minute contact time (5). Given these parameters, eradication of this parasite can be accomplished readily from a depopulated farm.

Nitarsone, a feed additive, has been used to prevent *C. anatis* infection in turkeys in the United States. The efficacy of this prevention program is uncertain. Feeding nitarsone prior to the inoculation of turkeys with *C. anatis* did not prevent infection, and no differences were observed in trophozoite numbers in treated and control poults (26). Other experimental studies suggest that nitarsone can significantly decrease the parasitic burden of infected poults during a 2-week experimental period (3). In this study, production losses were only slightly ameliorated using this feed additive.

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Histomoniasis (Blackhead) and Other Protozoan Diseases of the Intestinal Tract

Larry R. McDougald

Introduction

Histomoniasis affects mainly the ceca and liver of many gallinaceous birds, although it may be found in the bursa of Fabricius, kidney, spleen and other tissues (36). Morbidity and death occur as a result of liver damage. Caused by the protozoan *Histomonas meleagridis*, the disease is characterized by ulceration and inflamation of the cecal walls, engorgement of the ceca with large caseous casts, inflamation of the mesenteries, and severe necrosis of the liver. It has been called infectious enterohepatitis or blackhead. The signs leading to the use of the term *blackhead* are neither pathognomonic nor distinctive because many other diseases may produce a similar appearance (Fig. 28.11A). The roles of the cecal worm (*Heterakis gallinarum)* as an intermediate host, earthworms as accessory hosts, and the ability of the organism to colonize new hosts by cloacal contact comprise one of the most intriguing relationships in parasitology. Research on histomoniasis became neglected after the discovery of highly effective control measures in the late 1960s, leaving considerable basic biological and biochemical work undone.

Economic Significance

Annual losses from mortality in turkeys has been estimated to exceed 2 million dollars. Other types of poultry also suffer losses. Decreased production from morbidity, loss of flock uniformity and chemotherapy expense increase the cost of production of broiler breeder hens. Although histomoniasis is usually less severe in chickens, losses from morbidity and mortality are estimated to be greater in chickens than in turkeys because of the frequency of occurrence and the numbers of birds involved (1). Clinical blackhead disease in broiler breeder pullet flocks reached epidemic proportions in the mid-late 1990s and continues as a severe threat to production of breeder hens. Outbreaks of histomoniasis in leghorn pullets in Georgia and Florida caused up to 20% mortality and high morbidity. Chicken houses may become badly contaminated by *Heterakis* worm eggs, causing outbreaks in flock after flock. In many instances, poultry companies have discontinued the use of individual farms because of intractable contamination with *Heterakis* and *Histomonas.* The nitroimidazole antihistomonals were disallowed by the Food and Drug Administration in the 1990s, leaving no products available for treatment of blackhead disease. Histomoniasis remains an important limit to productivity in chickens, turkeys, and other fowl. This disease has no public health significance, as it affects only birds.

History

Histomoniasis in turkeys was first described in 1895. The literature has been reviewed in depth (23, 28, 34). Discovery that

chickens suffered a milder form of the blackhead, and often remained carriers resulted in the first useful recommendation for control: Turkeys should not be reared with chickens or on a range where chickens have been produced during the previous several years. The role of cecal worms (*Heterakis*) and earthworms as intermediate hosts for *Histomonas* explains this long period of infectivity on uninhabited range.

Tyzzer described the parasite as a simple cell with flagella as well as pseudopodia and published extensive observations on its biology (38, 39). The complicated nature of the pathogenesis of histomoniasis was described in detail between 1964 and 1974 by studies showing that certain bacteria are necessary in addition to the histomonads to produce disease. This interesting *Histomonas*-bacteria connection was discovered using germ-free techniques at the Universities of Georgia and Notre Dame. More recently, it was found that *Eimeria tenella*, the cecal coccidium, contributes significantly to the development of liver lesions in the chicken (27), and that histomoniasis is able to spread from bird to bird by means of cloacal contact with fresh droppings.

Etiology and Classification

The causative agent is *H. meleagridis,* a flagellated ameboid protozoan of about 10 μm. A larger (17 μm), nonpathogenic, four-flagellated histomonad found in the cecum was named as a separate species, *H. wenrichi.*

The disease is commonly called blackhead, after the mistaken impression that birds commonly developed a blackened head sooner after death than with other diseases. Other common names include histomoniasis and enterohepatitis.

Other agents, such as trichomonads and fungi (*Candida albicans*), have been advanced as etiologic agents of blackhead (34). However, these organisms are causing separate, unrelated diseases. The term *pseudo-blackhead* has been sometimes used to describe cases that did not respond to antihistomonals, but this term has little diagnostic value. Differential diagnosis used to resolve questionable infections include microscopic observation, histopathology and culture of responsible organisms.

Morphology

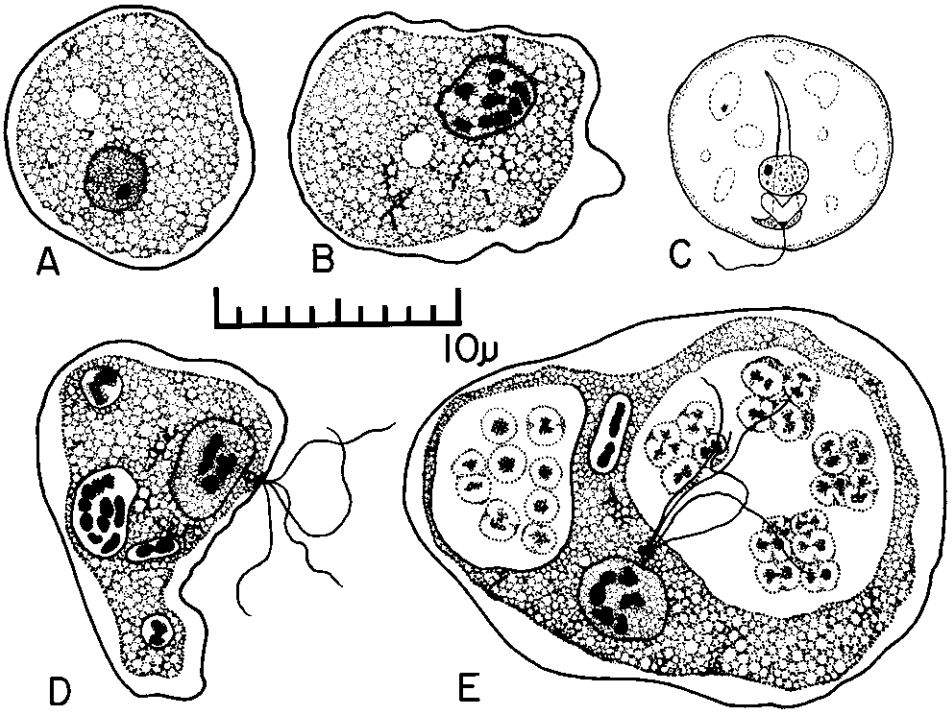
H. meleagridis in its nonamoeboid state is nearly spherical, about 10 μm (3–16 μm) in diameter. The amoeboid phase is highly pleomorphic. Pseudopodia may be observed if the slide is warmed during microscopy (see Fig. 28.12). Cecal lumen forms have a single flagellum 6–11 μm in length. A pelta and an axostyle are wholly contained within the cell. The parabasal body is V shaped and anterior to the nucleus. The nucleus is spheroid to ellipsoid or ovoid and averages 2.2 × 1.7 μm. These organisms lack mitochondria, instead relying on other organelles (hydrogenosomes) for energy metabolism. Hydrogenosomes are only observed by means of electron microcrospy.

The tissue forms usually lack flagella and reportedly exist in several different forms: 1) Parasites in the invasive stage at the peripheral areas of the lesions are 8–17 μm in size, amoeboid, and appear to form pseudopods. 2) A vegetative stage is larger (12–21 μm) and more numerous and is clustered in vacuoles in degenerating tissue. 3) A third stage present in older lesions is eosinophilic and smaller and may represent a degenerating form.

Life Cycle

The Role of Heterakis gallinarum

The survival and transmission of this organism is intimately associated with the cecal nematode *H. gallinarum* (38). It is doubtful that any other organism or mechanism is responsible for sur-



28.12. Examples of *H. meleagridis* (A, B, C) compared with *H. wenrichi* (D, E) showing variations for each species. A. Tissue type *H. meleagridis* in fresh preparation from liver lesion; viewed with phase-contrast microscopy. B. *H. meleagridis*; transitional stage with pseudopodia but no flagellum, in lumen of the cecum. Distribution of chromatin suggests the beginnings of binary fission. C. An organism from culture, with free flagellum typical of lumen-dwelling forms. (Honigberg and Bennett) D. Small *H. wenrichi*, structurally distinguishable from *H. meleagridis* by its larger size and by presence of 4 flagella. E. *H. wenrichi* as viewed in stained smear from cecum in which packets of *Sarcina* were abundant. Drawn from living specimens (A, B, D, and E) or tissue sections.

vival of histomoniasis from one flock to the next. Early attempts to find the histomonad in cecal worm eggs were inconclusive until Gibbs (10) demonstrated small bodies seen with the light microscope. Lee (19) observed a small form (3 μm) by electron microscopy, and histomonads have been cultured *in vitro* from heterakid eggs (35).

Histomonads are found in intestinal epithelial cells of very young worms or newly hatched larvae. The mechanism of egg infection by histomonads has not been determined. Springer *et al.* (36) found that triturated male worms recently removed from chickens carry viable histomonads. Female worms are less likely to transmit viable histomonads until the eggs within them mature. Unembryonated eggs are unlikely to transmit the infection. The female worms probably become infected with the histomonads during copulation and incorporate the protozoan into eggs before shell formation.

In the ceca of its host, the histomonad leaves the worm larva and multiples in the lumen and mucosa. Within 2–3 days the tissue forms enter the bloodstream and are carried to the liver by the hepatic-portal system. In the cecal tissues and in the liver the cells divide and grow, forming necrotic areas that are visible on gross inspection. Infection of other organs sometimes occurs, including the bursa of Fabricius, the kidney, the pancreas, and the spleen. The DNA of histomonads is also found in other tissues where lesions are not seen.

The Role of Earthworms

Earthworms can serve as transport hosts in which heterakid eggs hatch and survive. The larvae persist indefinitely in tissues in an infective state (21). The earthworm, thus, serves as a means for collection and concentration of heterakid eggs from the poultry yard environment.

Earthworm transmission of *Histomonas* to the ringnecked pheasant (*Phasianus colchicus torquatus*) has been of documented importance in a partridge-pheasant histomoniasis outbreak at a game-rearing station in central Iowa (18). On range or pastures, where climate and soil types favor survival of heterakids and earthworms, the latter must be considered in attempts to control a recurrent histomoniasis problem.

Transmission by Direct Contact

Transmission of blackhead within a turkey flock occurs readily by direct contact between susceptible birds and infected birds or fresh droppings and does not require an intermediate host (12, 13, 15). These findings illustrate how an outbreak can spread through a flock of turkeys in a week or two, killing most of the birds. In chickens, there is little evidence that infections spread in this way, placing more emphasis on contamination of the soil with cecal worm eggs (11).

Although direct infection of turkeys by oral ingestion of viable histomonads in fresh droppings or diseased tissues is possible, their extremely delicate nature makes this route rather unlikely. Histomonads cannot survive long outside the host unless protected by the heterakid egg or earthworm. Several studies showed that oral ingestion of organisms in feces or from cultures did not produce infections in turkeys unless the acidity of the crop was neutralized.

Pathogenicity

Characteristics of the definitive host influence clinical manifestations of infection by *Histomonas meleagridis* more than variations of pathogenicity of the parasite. These characteristics include species, breed, and intestinal flora.

Although naturally occurring infections occur in several species, the turkey is considered the most susceptible host because most infected turkeys suffer morbidity and death. Chickens are easily infected but often have a milder form of the disease. Outbreaks lead to an increase in mortality, culling, and loss of flock uniformity. Some other birds (peacocks, pheasants, etc.) are also severely affected. Variation in susceptibility has been found among different breeds and strains of chickens. Chickens 4–6 weeks old are most susceptible to infection, although turkeys of any age are highly susceptible. The 1990s rash of clinical outbreaks in broiler breeder pullets were unexplained, but it was suspected that concurrent outbreaks of immunosuppressive viral infections contributed by depressing T-cell responses.

Bacterial flora are also important in the development of blackhead disease. Lesions of histomoniasis were not produced in germfree turkeys or chickens unless bacteria were introduced (37). Disease was produced when *Clostridium perfringens*, *Escherichia coli, B. subtilis,* or mixed cultures were present. Isolates of *Histomonas* grown *in vitro* frequently lose pathogenicity in successive passages (6, 7). This fact discouraged early workers from attempting vaccine development using attenuated clutures because there was no way to stabilize the degree of attenuation (39). Although field strains with variable pathogenicity have been found, none has been characterized, with the exception of *H. wenrichi,* now listed as a separate species.

Susceptibility to Physical and Chemical Agents

The naked protozoan is not resistant to environmental conditions outside the bird. Thus, it is not important to practice extensive decontamination, even after an outbreak. Much attention is given to control of the intermediate host, the cecal worm. This is more important in chickens than in turkeys, because chicken farms are often contaminated with cecal worms. Turkey farms are rarely contaminated with histomonad-bearing worms, otherwise every flock would suffer blackhead outbreaks. Instead, outbreaks occur from the fresh introduction of worm eggs into a facility, usually on the shoes of the workers.

Control of worms depends on frequent application of benzamidazole-type wormers. Longer treatment is necessary (2–3 days) for a high degree of worms control. Some veterinarians recommend litter or soil treatment to reduce contamination by worm eggs, but there is no experimental evidence that this is effective.

Incidence and Distribution

There have been no surveys to describe the prevalence of histomoniasis. However, the disease probably occurs wherever suit-

able avian hosts exist. In general, it is more prevalent in areas favoring the coexistence of the cecal worm *Heterakis gallinarum*, but it is regularly reported by diagnostic laboratories in the United States, Canada, and Mexico (1). Recent outbreaks in Europe emphasize the international prevalence of this disease. The scientific literature includes many reports from Asia. Outbreaks in turkeys tend to be sporadic, while exposure in chickens may be chronic. Most of the commercial breeder pullet facilities in North America are thought to be contaminated with *Heterakis,* which are potent reservoirs for histomoniasis. Game birds reared in captivity and some wild birds also serve as reservoirs.

Pathogenesis and Epidemiology

Natural and Experimental Hosts

Numerous gallinaceous birds are reported as hosts for *H. meleagridis*. The turkey, chicken, chukar partridge, peacock, pheasant and ruffed grouse may be severely affected, while guinea fowl, bobwhite quail, and ostrich have a milder form of the disease. Ducks may become asymptomatic carriers. The coturnix quail can be infected experimentally, but it is a poor host.

Vectors

The common cecal worm, *Heterakis gallinarum*, is the only worm known to serve as an intermediate host for blackhead (38). Even closely related nematodes are unable to serve as hosts. Worm ova are resistant to environmental conditions and may remain infective for 2–3 years. Most gallinaceous birds are host to the cecal worm, and wild populations may serve as reservoirs. The common earthworm has been shown to consume and harbor infective larvae of the cecal worm, thus serving as a vector. While poorly documented, arthropods such as flies, grasshoppers, sowbugs and crickets may serve as mechanical vectors. Diagnosticians are sometimes confused by the inability to find cecal worms in birds with histomoniasis. Some of the reasons for this are that the cecal worm remains in larval form for 2–3 weeks and is very small (2–3 mm). Also, the development of histomoniasis destroys the environment for the worm, so that many are killed or expelled.

Incubation Period

Disease is caused when histomonads penetrate the cecal wall, multiply, enter the bloodstream, and eventually parasitize the liver. Overt signs of histomoniasis are apparent from 7–12 days and occur most commonly 11 days postinfection (PI). The infection begins with reddening and thickening of the cecal mucosa within 3 days. The incubation period varies with the size of the infective dose. Infections from worm eggs require longer than those beginning from cloacal exposure within an outbreak. Experimentally, cecal and liver lesions in turkeys develop about 3 days earlier with cloacal inoculation compared with infection via heterakid eggs. Once an infection is established in a turkey facility from introduction of cecal worm ova, the outbreak may spread rapidly by direct contact. Turkeys become infective to others within 2–3 days after becoming infected.

Clinical Signs

Signs of histomoniasis in turkeys (Fig. 28.11B) include yellow feces, drowsiness, dropping of the wings, walking with a stilted gait, closed eyes, head down close to the body or tucked under a wing, and anorexia. Sick birds tend to huddle together. The head may or may not be cyanotic, a sign observed by those who gave the disease the name blackhead. About 6–12 days PI, turkeys become emaciated. Infections in chickens may be mild and go unnoticed or may be severe and cause high mortality. Sulfur-colored droppings are seen in the later stages of disease when liver function is severely damaged and bile pigments are excreted through the kidneys. In chickens, the main signs are drooping feathers, morbidity, and closed eyes. Cecal discharges may contain blood, and caseous cecal cores are common. Sometimes gross pathology of blackhead in chickens may resemble cecal coccidiosis.

Clinical Pathology

In chickens, total leukocytes are increased, composed mainly of heterophils, lymphocyte, and basophils. Hemoconcentration occurs with severe dehydration.

In turkeys there is a decline in serum nitrogen, uric acid, and hemoglobin levels during the incubation period, but these return to normal prior to death. Blood sugar levels rise during the cecal phase, decrease during liver lesion development, and drop below normal prior to death. Serum albumin falls very low, but the globulins increase significantly during the acute infection in turkeys (25).

Plasma levels of glutamic oxaloacetic transaminase (GOT) and lactic dehydrogenase (LDH) increase as liver lesions develop in turkeys (26), but glutamic pyruvic transaminase (GPT) remains essentially unchanged. There is very little GPT activity in avian liver or other tissues, suggesting that it is not an important enzyme in birds. Appearance of a brilliant yellow urine pigment coincides with depressed liver function and elevated enzymes resulting from tissue damage. In acutely ill turkeys, the proportion of hemoglobins in the methemoglobin state in the blood is greatly elevated, possibly contributing to cyanosis and the purported blackhead appearance.

Morbidity and Mortality

The degree of illness is variable especially in chickens and other birds. Turkeys are more uniformly affected, with flock mortality commonly 80–100%. The time of onset in chickens varies. Blackhead may be diagnosed at 6–7 weeks of age in a flock, then at earlier ages in successive flocks as the disease reservoir becomes established in a facility. Outbreaks are seen as early as 3 weeks. Farmer and Stephenson (8) reported that turkeys confined to areas contaminated by chickens suffered 89% morbidity and 70% mortality. Although losses from histomoniasis in chickens are generally low, mortality has exceeded 30% in some naturally occurring infections. Mortality may occur as early as 6 days PI in experimental infections, but is most common at 9–12 days PI. Occasionally, a strain of *Histomonas* with apparent high virulence for chickens is found. There are no reference strains of *Histomonas*, precluding easy study of this phenomenon.

Gross Lesions

The primary lesions of histomoniasis develop in the ceca and liver (see Fig. 28.11). In recent years, lesions are also reported from other organs, such as the spleen, the bursa of Fabricius, pancreas and kidneys (36). Lesions are observed initially in the ceca. After tissue invasion by histomonads, cecal walls become thickened and hyperemic. Serous and hemorrhagic exudate from the mucosa fills the lumen of ceca and distends the walls with a caseous or cheesy core, and ulceration of the cecal wall may lead to perforation of the organ and cause generalized peritonitis.

Liver lesions in turkeys are often apparent a few days after infection and are highly variable in appearance. Often, the lesion is described as a circular depressed area of necrosis up to 1 cm in diameter and is circumscribed by a raised ring. Although these lesions are often seen (Fig. 28.11C,F), they may take on other appearances. In heavy infections, lesions may be small, numerous, and mostly subsurface, and they may involve a large part of the liver. In rare cases of recovery, lesions leave purulent scars on the surface of the liver. The liver may be enlarged and discolored green or tan. Lesions in lung, kidney, spleen, and mesenteries are sometimes recognized as white rounded areas of necrosis.

Histopathology

Initial invasion of the cecal wall results in hyperemia and heterophil leukocyte infiltration, probably a combined response to bacteria, histomonads, and heterakid juveniles (2). Within 5–6 days, numerous histomonads are visible as pale, lightly stained, ovoid bodies within lacunae in the lamina propria and muscularis mucosa. Large numbers of lymphocytes and macrophages have infiltrated tissues by this time, and the heterophil population has also increased. Cecal cores are composed of sloughed epithelium, fibrin, erythrocytes, and leukocytes along with trapped cecal ingesta. The core initially may be amorphous and red tinged, but by about 12 days, it appears laminated, dry, and yellowish from buildup of successive layers of exudate. By 12–16 days, giant cells appear in the tissues of the cecum. Coagulation necrosis and histomonad invasion extend well into the muscular tunic, extending nearly to the serosa. In survivors, histomonads are scarce within the tissues by 17–21 days and are mostly concentrated near the serosal layers. Large numbers of giant cells form and may appear grossly as granulomata bulging upon the serosal aspect of the cecum. Old lesions, after recovery, are characterized by lymphoid centers scattered throughout the cecal tissue. Expulsion of cores and the regeneration of epithelium may occur, particularly in chickens, but the cecum may suffer permanent damage.

The liver has microscopic lesions visible by 6–7 days PI and consisting of small clusters of heterophils, lymphocytes, and monocytes near portal vessels. Histomonads are difficult to visualize in these areas. After 10–14 days, the lesions are enlarged, becoming confluent in some areas. There is extensive lymphocytic and macrophage infiltration, and heterophils are present in moderate numbers. Hepatocytes in centers of the lesions necrose and disintegrate. Many individual or clustered histomonads are visible in lacunae near the periphery of lesions. From 14–21 days PI, necrosis becomes increasingly severe, resulting in large areas consisting of little more than reticulum and cellular debris. Histomonads at this stage are present mostly as small bodies in macrophages. If recovery occurs, foci of lymphoid cells remain, along with areas of fibrosis and regenerating hepatocytes.

Immunity

Immunity to blackhead has not received much study, and most of the reports on this topic are more than 50 years old. Early work suggested that a partial immunity developed after infections, which might or might not be adequate to protect against reinfection (3, 39). Most work with immunity in turkeys has relied on drug treatment to terminate infections, because turkeys usually die from the disease.

Attempts to immunize chickens and turkeys with histomonads attenuated *in vitro* have been only partially successful. Some protection was demonstrated against cloacal inoculation of pathogenic or attenuated strains of histomonads, but very little against histomonad-containing heterakid eggs (22). Although reports are not in agreement, some protective immunity is obtained while drug therapy is being administered. Research on the use of attenuated strains for immunization was abandoned because it was considered impossible to stabilize the degree of attenuation *in vitro* and impractical to inoculate birds with the live cultures.

Chickens and turkey produce antibodies against *H. meleagridis* antigens prepared from infected livers and ceca, and after natural or experimental infections. In some reports, antibodies in turkeys and chickens did not confer resistance to reinfection. Such antibodies persisted for a considerable time (3). Birds recovering from histomoniasis may harbor parasites in the ceca without signs or lesions of the disease (5).

Recent experimental work suggests that infected birds develop precipitins and lysins in the serum, and that birds given injections of *H. meleagridis* antigen may develop good protection against reinfection.

Passive Immunity

Attempts to transfer immunity from resistant to susceptible chickens and turkeys by repeated intraperitoneal injections of serum from immune birds have been unsuccessful. When birds receiving immune serum were challenged by cloacal inoculation of infected liver homogenates, turkeys died from histomoniasis, and all chickens developed typical cecal lesions (4, 5).

Diagnosis

Most experienced poultry workers make a field diagnosis in turkeys on the basis of gross appearance of lesions. Laboratory confirmation is sometimes necessary to rule out concurrent infections with other agents that affect the cecum or liver (coccidiosis, salmonellosis, aspergillosis, and upper digestive tract trichomoniasis). Chickens are more likely to have lesions in the ceca that can be confused with other diseases, particularly cecal coccidiosis.

The presence of characteristic lesions in turkeys is sufficient for presumptive diagnosis. Identification of histomonads by microscopy adds confidence to the diagnosis. The organisms are most easily observed with phase-contrast microscopy with fresh specimens. Histomonads remain active and are more easily iden-

tified if the microscope stage is warmed, either with a special stage incubator or small incandescent light bulb. Tissue forms are not easily identified by microscopy.

For routine diagnostic histopathology, any of several stains, including hematoxylin and eosin or periodic acid-Schiff, may be used (18). Excellent cytologic preparations have been made from fresh cultures using Hollande's cupric picroformol and a protein-silver stain.

Where freshly killed birds are available, it is a simple matter to cultivate histomonads *in vitro* as a diagnostic aid, using Dwyer's medium (6,24). If samples are taken from freshly killed birds the test is more than 75% accurate. The medium consists of 85% Medium 199 in Hank's balanced salt solution, 5% chicken embryo extract (CEE50), and 10% horse or sheep serum, adjusted to pH 7.2. A small amount (10–20 mg) of rice powder (organically produced rice flour from a health food store) is added, and then the tubes are sealed, incubated at 40°C overnight, and observed with an inverted microscope. Cultures obtained in this way can be maintained by subculturing every 2–3 days, and can be used to infect birds experimentally, but they tend to become nonpathogenic within 6–8 weeks.

While it is not usually necessary for diagnosis, PCR tests are highly accurate in identification of *H. meleagridis*. Recent tests rely on primers based on the small-subunit RNA fraction (21).

Prevention and Control

As there are no chemotherapeutic products available for treatment of infections, and there is no commercial vaccine for histomoniasis, control measures are focused on prevention.

The primary reservoir of infection is the cecal worm ova. Thus, prevention is largely a matter of avoidance of contact of susceptible birds with sources of egg contamination and prevention of exposure to carriers.

For the protection of turkeys, exclusion of chickens from any contact is essential, because chickens may often harbor large numbers of egg-laying cecal worms. Outdoor turkey ranges can become contaminated with heterakid eggs, creating a situation in which histomoniasis recurs in turkey flocks for many years. Because of the longevity of infectious eggs, range rotation is not practical as a solution.

Rearing turkeys indoors tends to reduced outbreaks of blackhead, but exacerbates the extent and severity of outbreaks. It is most likely that outbreaks arise by the introduction of a small number of cecal worm ova into the growing facility. After introduction, the infection spreads throughout the flock by direct contact. The recent observation that histomoniasis cannot spread within a flock without direct contact between birds offers a potential method for containing outbreaks. If the growing facility is divided into subunits, even by netting or other barriers, the outbreak will be limited to the contaminated units.

Leghorn pullets and broiler breeder pullets often become infected in problem houses where worm eggs have built up in number for several years. In some areas histomoniasis is reportedly common in broilers. In some instances, disinfection may have value in killing worm eggs, but there is no experimental work to support this conclusion.

The discovery that cecal coccidiosis (*E. tenella*) interacts with histomoniasis in chickens is important because coccidia are often present in growing facilities. In experimental tests, the number of birds showing liver lesions, as well as the severity of lesions, was increased when both parasites were present, even when low infections of coccidia were used (27). These results suggest that better prevention of coccidiosis in breeder and layer pullets is important in avoiding problems with blackhead.

Chemotherapy

Management practices alone are rarely adequate to keep the disease at a low level in commercial flocks; therefore, preventive chemotherapy is sometimes practiced during the high-risk part of the growout. Presently, the only product available for the prevention of histomoniasis in poultry is nitarsone (Histostat7, Alpharma, Clifton, NJ).

Five drugs were at one time registered for treatment of outbreaks in the United States (9), including 2 arsenicals, 2 nitroimidazoles, and 1 nitrofuran (Table 28.5). However, regulatory action has removed the most useful drugs (nitroimidazoles) from the market. These products are also unavailable in other countries. Preventive use of nitarsone is usually effective in keeping blackhead at a low level, but its use in breeder stock presents additional problems. Breeder pullets and males are commonly reared on skip-a-day feeding programs, which lead to exacerbated toxicity of the arsenic. The arsenicals are not strong enough to be effective as treatment drugs and are not approved for this use. For a historical review of older literature on antihistomonal drugs, see Joyner *et al.* (17) and Joyner (16).

Because of the close association of *H. meleagridis* with bacteria, it has become common practice to treat outbreaks with antibiotics. While it is generally beneficial to administer antibiotics to combat secondary infections, there is no evidence that such treatments have a direct effect on histomoniasis (14).

Worm control is considered a central part of blackhead control programs for chickens. Frequent worming with benzamidazole type anthelmintics is known to reduce exposure to both worms and histomonads. It is important to administer wormers at least one week prior to the usual expected time of outbreaks, based on the history for each farm.

Trichomoniasis

Introduction

Trichomoniasis in birds, affecting the upper digestive tract, is caused by the flagellated protozoan *Trichomonas gallinae* (Fig. 28.13). In pigeons, it causes a condition known as *canker*. Turkeys, chickens, and a wide variety of wild birds are parasitized with varying degrees of pathogenicity (20).

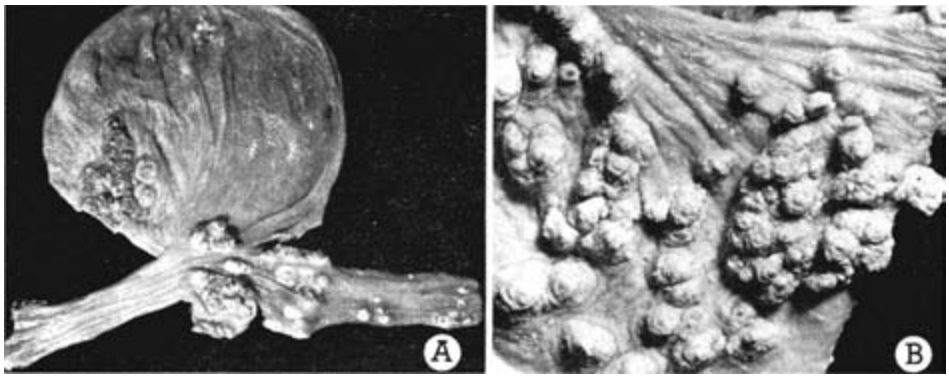
Description

These intestinal flagellates are rapidly moving, pear-shaped protozoa that range in size from 5–9 μm in length and from 2–9 μm in width (Fig. 28.14). There are typically 4 free flagella arising

Table 28.5. Feed additives or water treatments at one time registered in the United States for the prevention or treatment of blackhead disease in turkeys.[a]

			Conditions of Use		
Drug	**Trade Name**	**Supplier**	**Use Level**	**Withdrawal**	**Approval for Chickens**
Carbarsone[b]	Carb-O-Sep	N/A	0.025–0.037%	5	No
Dimentridazole[b]	Emtrymix	N/A	0.015–0.02%[c] or 0.16–0.08%[d]	5	No
Furazolidone[b]	nf-180	N/A	0.011%[c]	5	Yes
	Furox	N/A	0.022%[d]	5	Yes
Ipronidazole[b]	Ipropran	N/A	0.00625%[c]	4	No
			0.0625%[c]	4	No
Nitarsone	Histostat-50	Alpharma	0.01875%	5	Yes

[a]Some products were also available for water treatment.
[b]Only nitarsone remains available in the USA.
[c]Preventive level.
[d]Treatment level.



28.13. A. Necrotic ulceration of the esophagus and crop seen in trichomoniasis. B. Necrotic ulcers characteristic of trichomoniasis of upper digestive tract. Note pyramidal shape of tissue (Hinshaw and Rosenwald).

from a basal granule at the anterior pole of the organism. A slender axostyle usually extends well beyond the posterior end of the body. An undulating membrane originates at the anterior pole of the body and ends short of the posterior pole, with the enclosed flagellum not trailing free at the posterior end. The flagella and internal structures can be seen only with the aid of phase-contrast microscopy or special stains.

Incidence and Distribution

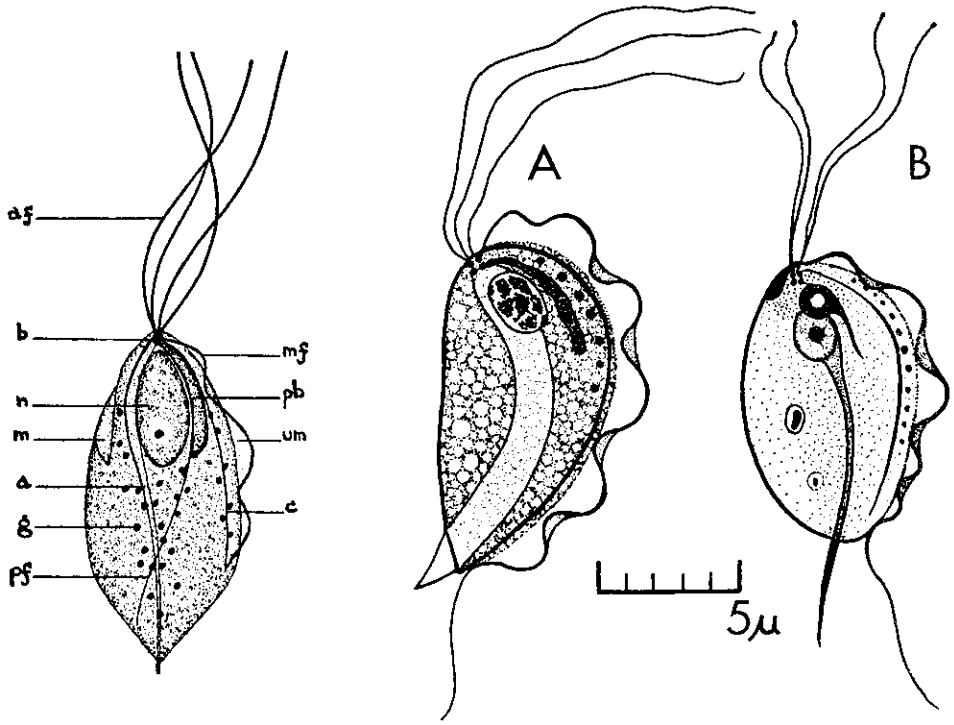
Squabs usually become infected with their first taste of "pigeon milk" from the crop of adults and usually remain carriers throughout life. With virulent strains, mortality may be as high as 50% before sufficient protective immunity develops. Pigeons are often blamed for transmission of trichomoniasis to turkeys and chickens. The economic impact of the disease in turkeys and chickens is difficult to assess, although infections are occasionally reported. When captive birds of prey such as falcons are allowed to feed on pigeons, infection may result in a condition known as "frounce" among falconers.

Life Cycle

T. gallinae reproduces by longitudinal binary fission. Cysts, sexual stages, or vectors are not known. The organism is transferred to squabs by infection of "pigeon milk" from adults. In chicken and turkey flocks, infection is spread by contamination of drinking water and perhaps feed.

Pathogenesis and Pathology

Nearly all pigeons are carriers of this organism. The virulence of *Trichomonas* varies widely, with some strains capable of causing mortality. At one time investigators considered trichomoniasis to be synonymous with blackhead disease. However, these investigators failed to consider that more than one parasite might produce lesions of similar appearance. Affected birds may cease to feed and become listless, ruffled in appearance, and emaciated before death. A greenish to yellowish fluid may be seen in the oral cavity and may drip from the beaks of infected birds.



28.14. *Trichomonas gallinae*, semidiagrammatic (left): (a) axostyle, (af) anterior flagellum, (b) blepharoplast, (c) costa, (g) cytoplasmic granules, (m) mouth, (mf) marginal filament, (n) nucleus, (pb) parabasal body, (pf) parabasal fibril, (um) undulating membrane (Stabler). Two common trichomonads of the lower digestive tract of domestic birds (right), as specimens fixed in Schaudinn's fluid and stained with Heidenhain's hematoxylin may appear. A. *Tritrichomonas eberthi*. B. *Trichomonas gallinarum*. (Lund)

Gross Lesions

T. gallinae invade the mucosal surface of the buccal cavity, sinuses, pharynx, esophagus, and crop and occasionally the conjunctiva and proventriculus. The liver is frequently invaded, and occasionally other organs—but not the digestive tract below the proventriculus—are involved.

Lesions appear initially as small, circumscribed caseous areas on the surface of the oral mucosa, which may be surrounded by a thin zone of hyperemia. These may enlarge and become confluent. The buildup of caseous material may be sufficient to occlude the lumen of the esophagus partially or completely. These lesions eventually may penetrate tissue and extensively involve other regions of the head and neck, including the nasopharynx, orbits, and cervical soft tissues. In the liver, lesions appear on the surface and extend into the parenchyma as solid, white to yellow circular or spherical masses.

Histopathology

Pigeons infected with a virulent strain of *T. gallinae* had purulent inflammation with caseous necrosis as the predominant lesion (33). Trichomonads multiply in secretions and on the mucosal surface of the oropharynx. Ulceration of the mucosa with a massive inflammatory response, primarily heterophils, is well established by the fourth day of experimental infections. In the liver, focal necrotic abscesses occurred in all zones of lobules, with an inflammatory reaction characterized by mononuclear cells and heterophils. As liver lesions progressed, no intact hepatocytes remained in the center of foci; trichomonads were most numerous at the periphery.

Immunity

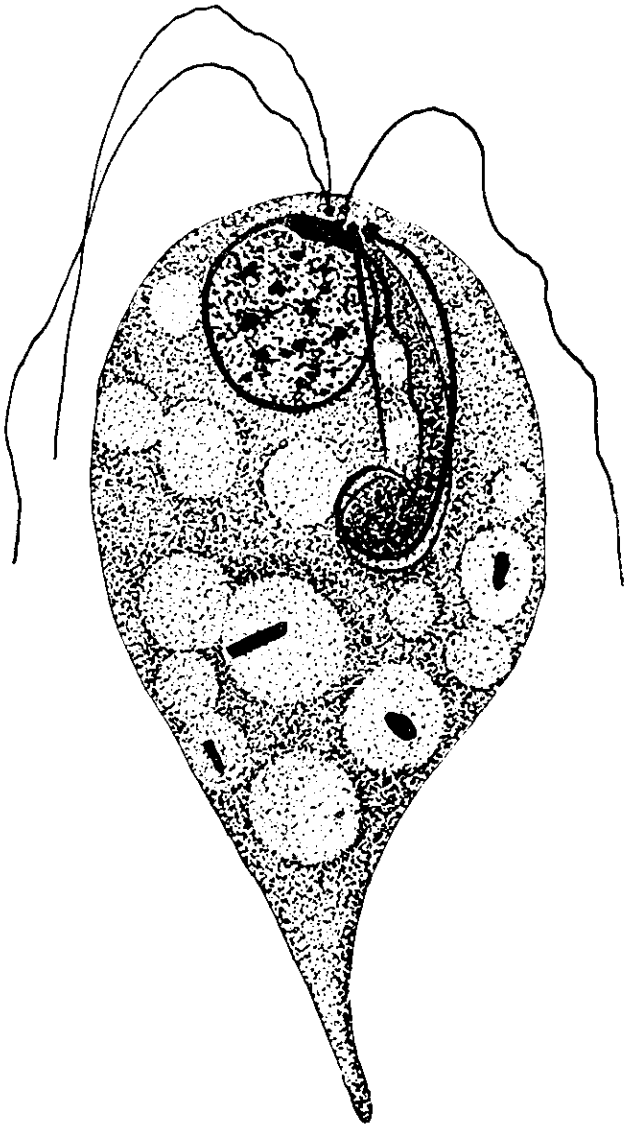
The relatively high incidence of infections in otherwise normal pigeons can be attributed to strain variations, acquired immunity, or both. Pigeons are immune to disease from virulent strains of trichomonads after recovery from sublethal trichomoniasis. Plasma from pigeons harboring any of 3 strains of *T. gallinae* could protect other pigeons against disease but not infection from a virulent strain.

Antigens of *T. gallinae* have been studied in regard to taxonomy with the conclusion that virulence and antigenic composition were related (7).

Diagnosis

Clinical signs and gross lesions are highly suggestive and may be confirmed by microscopic observation of organisms in direct wet smears from the mouth or crop. Histopathologic examination or cultivation of organisms in artificial media may help in cases in which the parasites are absent in fresh smears. Trichomoniasis must be differentiated from candidiasis and hypovitaminosis-A, which can produce somewhat similar lesions. History, cultivation for fungi, and histopathologic examination may prove useful in resolving problem diagnoses.

Several other species of flagellates that inhabit the avian gastrointestinal tract are frequently misidentified as *T. gallinae.* These other species of trichomonads and more distantly related flagellates have never been unequivocally demonstrated to be pathogenic for the avian host. Their recognition as harmless commensals will prevent unnecessary expenditures for therapeutic measures.



28.15. *Chilomastix gallinarum*, semidiagrammatic, illustrating details of morphology. (Boeck and Tanabe)

One trichomonad, *Tetratrichomonas gallinarum,* is a common inhabitant of the cecum of chickens and other gallinaceous birds. This trichomonad or a closely related species occasionally has been isolated from liver and blood. Although lesions have been ascribed to this organism, no confirmation of pathogenicity has come from experimental infection.

Other lower intestinal protozoa such as *Chilomastix gallinarum* (Fig. 28.15), a cyst-forming flagellate with a large cytostomal cleft but no undulating membrane, and *Cochlosoma anatis,* with a ventral sucker covering half the surface of the body, are apparently nonpathogenic. Although additional controlled experiments with flagellates found in the lower intestine are needed, for the present, they should not be considered important.

Prevention and Control

Because *T. gallinae* is transmitted from parent to squab in pigeons and by contamination of feed and water by oral fluids in the case of domestic fowl, sick birds should be removed from a flock. Drugs with activity against other related protozoa (*H. melelagridis, Entamoeba histolytica, Giardia lamblia)* are active against trichomoniasis in pigeons or turkeys, however none is approved for use in domestic birds. McLoughlin (31) found dimetridazole useful at a level of 0.05% in drinking water for pigeons. This drug is no longer available in the United States. There is no vaccine for this parasite.

Hexamitiasis

Etiology and Distribution

Hexamitiasis, or infectious catarrhal enteritis, of poults is caused by the protozoan *Spironucleus meleagridis.* There is apparently no well-known common name for this parasite other than the original generic name *Hexamita.* There is no good measure of economic losses from this parasite. However, the USDA estimated that an annual loss of $667,000 occurred from hexamitiasis in turkeys from 1942–1951 (40). Cases of hexamitiasis are encountered sporadically in diagnostic laboratories in the United States. The disease has been reported from several areas of the United States, Canada, Scotland, England, and Germany. The organism has also been found in pheasants, quail, chukar partridge, and peafowl, which may be a source of infection for range-reared turkeys. The 8 prominent flagella include 4 anterior, 2 anterolateral, and 2 posterior. The 4 anterior flagella are recurved along the body (Fig. 28.16). McNeil *et al.* (32), who named the species, described it as being 6–12.4 × 2–5 μm in size with binucleate large endosomes.

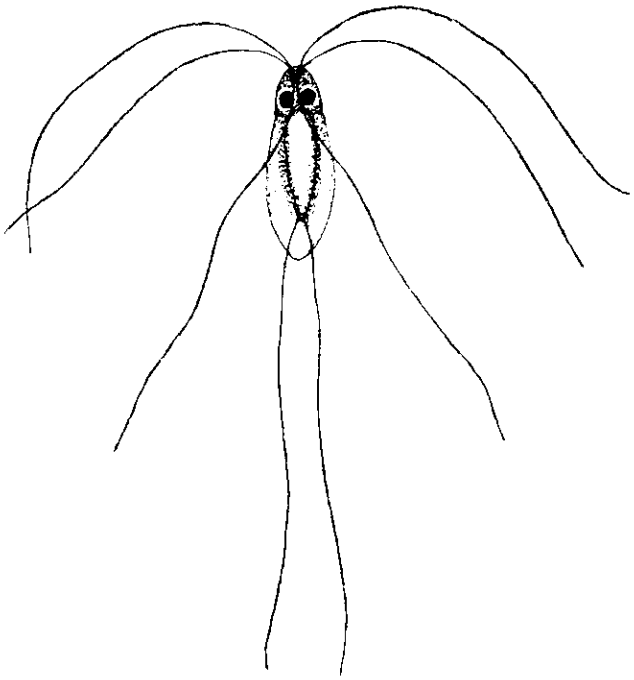
Pathology

Affected poults do not show specific signs, but a watery diarrhea occurs that may become yellowish later in the course of the disease. The poults at first are nervous and active but later tend to become listless and huddled. Convulsions and coma may occur as the terminal stage is approached.

Lesions include catarrhal inflammation and atony resulting in distention, especially in the upper small intestine. Intestinal contents are watery, and large numbers of *S. meleagridis* may be seen in the crypts upon microscopic examination. A yellowish discoloration of the liver surface was described in an outbreak in Germany.

Diagnosis

The presence of watery diarrhea and the microscopic demonstration of flagellated *S. meleagridis* in fresh smears of duodenal contents are sufficient to establish the diagnosis. Survivors may become carriers; thus, the parasites may be seen without any signs of infection. These parasites are easily distinguished from



28.16. *Spironucleus meleagridis* from the intestine of a turkey. (25)

other protozoa by their rapid, darting movement. They are small, in comparison with other flagellates.

Control and Treatment

There is no effective treatment, although butynorate (0.0375%) and chlortetracycline (0.0055%) were approved for use at one time. There is no vaccine for this parasite. The removal of carrier birds, the separation of older stock from poults, and the exclusion of other avian host species from the area of the poult flock are thought to minimize transmission. Attention to principles of good management is considered important in reducing losses to this and other parasitic diseases.

Miscellaneous Protozoa in the Digestive Tract

Several species of the genera *Entamoeba* and *Endolimax* occur naturally in the ceca or feces of various domestic fowl or can be established experimentally. Apparently, none of these are pathogenic; they exist by feeding on intestinal contents.

The amoebas have irregularly shaped trophozoites with a single nucleus with a more or less prominent endosome. They produce cysts containing 1, 4, or 8 nuclei. Phase-contrast microscopy or stained preparations is recommended for observing these organisms. A number of species have been reported (20, 30).

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Miscellaneous and Sporadic Protozoal Infections

Alex J. Bermudez

Leucocytozoonosis

This parasitic disease of birds affects blood and tissue cells of internal organs. Reviews of this and other parasitic diseases have been summarized by Lund (78), Levine (73), and Fallis *et al.* (33).

Leucocytozoon was assigned to the order Haemospororina of the phylum Apicomplexa (72, 76). Similarities in life cycle and ultrastructure of some life stages of *Leucocytozoon*, *Haematroteus*, and *Plasmodium* warrant inclusion of all 3 genera in a single family, Plasmodiidae (72, 73). Approximately 67 valid species and 34 synonyms have been described. With the exception of a species observed in the teiid lizard in Brazil, all species of *Leucocytozoon* are found in birds (48).

The life cycle requires 2 hosts; sporogony is in insects, although schizogony (merogony) and gametogony are in tissues or blood cells, respectively, of the vertebrate host. *Leucocytozoon* is prevalent in areas with a suitable ecology and ethology for dipterous invertebrate hosts, simuliid flies and culicoid midges. At least 3 species of *Leucocytozoon* reported in domestic fowl are known to cause outbreaks in North America resulting in economic losses in ducks, geese, turkeys, and chickens. Outbreaks of leucocytozoonosis are sporadic in North America (2), but the disease is relatively common in the open chicken houses of southern and eastern Asia (119), the Philippines, Indonesia, and eastern Africa (24).

The author wishes to acknowledge Wilfred T. Springer, who authored "Other Blood and Tissue Protozoa" in the 10th edition of *Diseases of Poultry,* for his substantial contributions to this current subchapter.

Leucocytozoon simondi *Mathis and Leger 1910*

Infection with *L. simondi* has been reported from 27 species of ducks and geese in United States, Canada, Europe, and Vietnam by Hsu *et al.* (48). *L. anatis* from ducks and *L. anseris* from geese are considered synonyms of *L. simondi*. Approximately 14–20% of ducks and geese along the northeastern seaboard of North America carry *Leucocytozoon* infections (8, 10). Eighty percent of geese at Seney Wildlife Refuge in Michigan had some parasitemia in 1963 just prior to the egg-laying season, and each year all goslings become infected (47).

Ducks and geese are suitable hosts for *L. simondi*, but chickens, turkeys, pheasants, and ruffed grouse are not.

Bloodsucking flies known to serve as the vector for *Leucocytozoon* in ducks include *Simulium venustum*, *S. croxtoni*, *S. euradminiculum*, and *S. rugglesi*.

Etiology

Sporogony occurs in the insect vector and may be completed in 3–4 days. Ookinetes develop following fertilization of the macrogametocyte and may be found in the stomach of the insect within 12 hours after a blood meal. Oocysts form from the ookinetes within the stomach of the invertebrate host and produce sporozoites, which migrate to the salivary glands after emerging from the oocyst. Viable sporozoites have been found in vectors up to 18 days after the last blood meal.

Schizogony takes place in internal organs of the vertebrate

host, such as liver, brain, spleen, and lungs. "Hepatic schizonts" in liver cells measure up to 45 μm when mature. Merozoites and syncytia are released from hepatic schizonts (syncytium refers to cytoplasm bounded by a plasma membrane and containing two or more nuclei). Some merozoites enter parenchymal cells of the liver and initiate another schizogonic cycle, and others enter erythrocytes or erythroblasts to develop into gametocytes. Syncytia are phagocytized by macrophages or reticuloendothelial cells throughout the body, where they develop into megaloschizonts up to 400 μm in size. Merozoites released from the megaloschizont enter lymphocytes and other leukocytes to form gametocytes.

The gametocytes of *L. simondi* found in the blood average 14.5 × 5.5 μm and usually inhabit elongate spindle-shaped host cells averaging about 48 μm in length. The parasite lies beside the nucleus of the host cell. Elongate gametocytes probably develop exclusively in leukocytes, predominantly lymphocytes and monocytes, and mature round gametocytes are found in erythrocytes. According to Allan and Mahrt (4), each *Leucocytozoon* species enters gametogony in only 1 type of host cell; therefore, the presence of 2 morphologic types in the same bird suggests a concurrent host infection with 2 species. Desser *et al.* (23) observed infections in some areas of northern Michigan that were characterized by presence of both hepatic schizonts and round gametocytes, which he attributed to different strains of *L. simondi*.

Gamonts may be differentiated with a Romanowsky stain based on the dark blue staining cytoplasm of the macrogamete with its red nucleus, and the very pale blue staining cytoplasm of the microgamont with its pale pink nucleus. The microgamonts are more delicate and subject to distortion (73).

Pathogenesis and Epidemiology

The pathogenicity of *L. simondi* in ducks and geese is well documented. An outbreak of *L. simondi* among ducks in Michigan resulted in 35% mortality. Extensive losses of young goslings, attributed to infections of *L. simondi*, are observed annually at Seney Wildlife Refuge, with mortality greater than 70% occurring every 4 years (47). However, not all *L. simondi* infections cause such severe disease. An experimental infection in anatid ducklings caused no mortality and no difference in growth rate (97).

Clinical signs vary with age and the condition of the host. Young ducklings manifest inappetence, weakness, listlessness, dyspnea, and sometimes death within 24 hours. Signs in adults appear less abruptly and consist of listlessness and low mortality. About 60% of fatalities occur 11–19 days postexposure. Some pathologic effects of the disease are anemia, leukocytosis, splenomegaly, and liver degeneration and hypertrophy. Extensive tissue damage was noted in the spleen and heart of ducks carrying megaloschizonts.

Kocan (70) described an anti-erythrocyte factor in sera from acutely infected ducks, which agglutinated and hemolyzed normal untreated erythrocytes as well as infected cells. This factor was believed to be a product of the parasite, and its action may account for the osmotic fragility of erythrocytes and anemia associated with *L. simondi* infections (79).

The greatest number of infections in northern Michigan occur in July. Gametocytes decrease in number in the blood until midwinter, when they disappear or become scarce and then reappear in the spring.

Leucocytozoon smithi *Laveran and Lucet 1905*

L. smithi was first seen in turkeys in the eastern United States by T. Smith, after whom it is named, and has since been reported in turkeys in North Dakota, Minnesota, Nebraska, California, Texas, Missouri, France, Germany, the Crimea, and Canada.

In the United States, it can be widespread in adult turkeys (105): 289 of 357 turkeys were found infected in Georgia, 60 of 67 in Florida, 4 of 12 in Alabama, and 7 of 9 in South Carolina. The incidence of infection in pen-raised and free-ranging mature wild turkeys in the Cumberland State Forest in Virginia was 100%. The incidence of infection in wild turkeys in South Carolina, Mississippi, and the midwestern states was 100%, 33%, and 3%, respectively (19, 36, 106). Economically significant outbreaks of *L. smithi* infection in turkeys are infrequent in North America (2), probably because of a transition to confinement rearing of commercial turkeys and the general shift away from raising turkeys in regions where blackflies and midges are abundant.

Etiology

L. smithi may be observed in the blood as rounded gametocytes that later become elongate, averaging 20–22 μm in length. They inhabit elongate cells averaging 45 × 14 μm, with pale cytoplasmic "horns" extending out beyond the enclosed parasite. The host cell forms a long, thin dark band along each side of the parasite. Gamonts are found only in leukocytes. The staining characteristics of the gamonts with a Romanowsky stain are similar to those of *L. simondi* (73) (Fig. 28.17A).

Intracellular schizogonous forms are found in the liver. Both schizonts and megaloschizonts were observed and illustrated by Siccardi *et al.* (98).

Several aspects of the life cycle were described in detail by Newberne and by Wehr (cited by 78); the ultrastructure of gametocytes was defined by Milhous and Solis (84). Gametocytogenesis, sporogonic development, schizonic development, fertilization, and ookinete differentiation have been described by light and electron microscopy (107, 108, 109, 110).

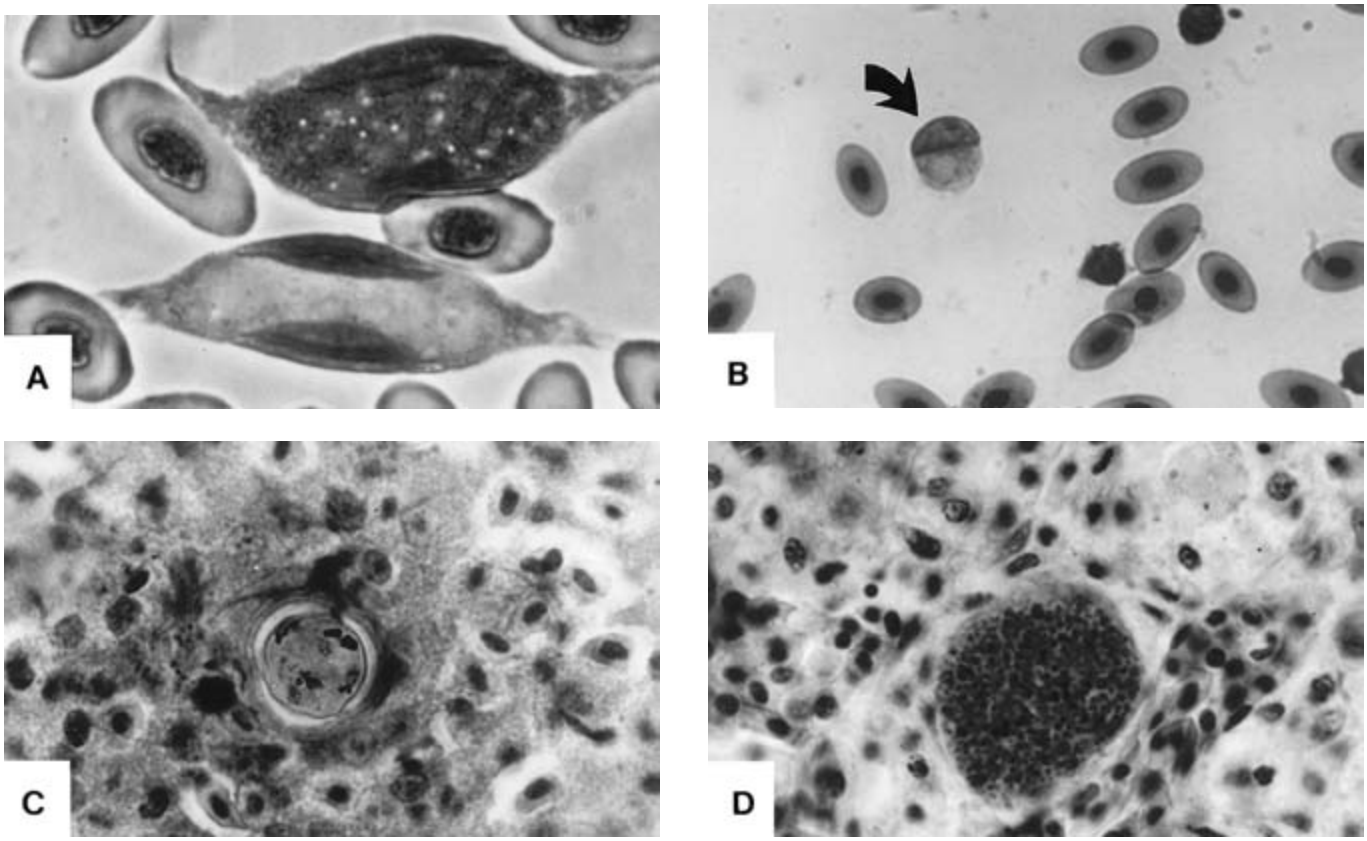
Pathogenesis and Epidemiology

L. smithi generally resembles *L. simondi* of Anseriformes, but turkeys are probably not susceptible to the latter. *L. smithi* is not transmissible experimentally to chickens or ducks.

Simulium occidentale, *S. aureum*, *S. meridionale*, *S. nigroparvum*, and *S. slossonae* have been listed as vectors for *L. smithi* (33, 69).

The progress of leucocytozoonosis in susceptible young turkeys may be rapid and fatal. Clinical signs include anorexia, excessive thirst, depression, somnolence, and sometimes muscular incoordination. Death may occur suddenly during the acute stage of the disease.

Heavy infections of *L. smithi* do not seem to occur in mature wild turkeys. Few signs of infection are observed in wild turkeys,



28.17. Turkey blood containing various stages of *Leucocytozoon smithi*. A. Darkly stained macrogametocyte (upper) and lightly stained microgametocyte (lower). Giemsa stain, ×1250. B. "Round form" often found early in infection (day 16), ×140. C. Megaloschizont in turkey liver; day 9. H & E. ×1000. D. Megaloschizont turkey kidney; day 10. (21)

possibly because of local factors such as time at which suitable vectors are prevalent and the age of birds at first exposure.

Domestic hens infected with *Leucocytozoon* had decreased egg production, egg weight, and hatchability, and higher mortality than uninfected hens (63).

Recovered birds may harbor the parasite in their blood for more than 1 year (24). There is often loss of vigor, and birds may suffer moist tracheal rales and coughing. Some birds die when subjected to stress. Males showed reduced mating activity (73).

Johnson *et al.* (61) reported that death results from obstruction of the circulatory system by large numbers of parasites. The lungs, small intestine, liver, and spleen may be congested, and the liver and spleen enlarged, in affected turkeys. Lund (78) cites extensive description of pathogenesis.

Leucocytozoon caulleryi *Mathis and Leger 1909*

L. caulleryi frequently is found in chickens in southern and eastern Asia. Infections occur frequently in Japan (86). Reports of leucocytozoonosis in South Carolina, probably caused by *L. caulleryi*, are the only known cases of the disease in chickens in North America. In one survey, 13.6% of domestic yard chickens in South Carolina were infected (92). *L. andrewsi* and *L. schueffneri* are considered by some protozoologists to be synonymous with *L. caulleryi* (73).

Etiology

The domestic chicken is the only reported host for *L. caulleryi*. Insect vectors are *Culicoides arakawa*, *C. circumscriptus*, and *C. odibilis*. Akiba's discovery that the vector was a species of *Culicoides* and not *Simulium* prompted some to place *L. caulleryi* in a new genus called *Akiba* (33). Leucocytozoonosis epizootics, widespread during summer months in Japan, are serious enough to cause deaths in growing chicks and reduced egg production in hens (86).

Pathogenesis

Early schizonts occur in the lung, spleen, and thymus (48). Megaloschizonts, often readily visible on gross examination, are found in numerous tissues including the liver, spleen, kidneys, pancreas, heart, lungs, proventriculus, ventriculus, intestines, and brain (42).

Mature gamonts are round and occupy round host cells, erythrocytes, and leukocytes about 20 μm in diameter. The nucleus of the host cell reportedly disappears after infection, a characteristic that differs from other species with round gametocytes. Macrogametes (12–15 μm) stain more darkly with Romanowsky stain than microgamonts (10–15 μm) , according to Levine (73).

Serious outbreaks of *L. caulleryi* in chickens are characterized by hemorrhage in the peritoneal cavity, perirenal hemorrhage, and subdural hemorrhage (42). Extensive hemorrhage in the kidneys and other tissues occurs when merozoites are released from mega-

loschizonts. In infected laying hens, the uterus is edematous on gross examination with schizont development, granuloma formation, and inflammation of the uterus noted on histopathology (91).

Leucocytozoon sabrezi *Mathis and Leger 1910*

L. sabrezi (*L. schueffneri*, probably a synonym) has been found in domestic chickens in Southeast Asia, causing anemia, thickened oral discharge, and paralysis of the legs. Megaloschizont formation has not been reported for this parasite. Merozoites enter both erythroblasts and leukocytes to form elongate gametocytes within spindle-shaped host cells (6–7 × 4–6 μm), whose nuclei appear as thin bands beside the parasite (24). Macrogametes (22 × 6.5 μm) have a more compact nucleus and stain more darkly with a Romanowsky stain than the microgamonts (20 × 6 μm) (53). The insect vector is unknown.

Leucocytozoon schoutedeni *Rodham, Pons, Vandenbranden, and Bequaert 1913*

L. schoutedeni, which was found in 50% of chickens in East Africa (24), is unknown elsewhere. Gametocytes are round (11–13 μm) and found in round host cells (18 μm) whose nuclei surround the parasite about one-half of its length. Staining characteristics of the gametocytes have not been reported. The *Simulium* fly serves as the invertebrate host for *L. schoutedeni*.

Diagnosis

Leucocytozoon infections are diagnosed by direct microscopic observation and identification of gametocytes in stained blood or schizonts in tissue sections. Solis (102) described the high staining contrast of *Leucocytozoon* in peripheral blood films stained with brilliant cresyl blue. A variety of serologic tests including the agar gel precipitation test, indirect IFA, ELISA test and latex agglutination test have been developed for detecting antibodies to *L. caulleryi* (54, 57).

Treatment and Control

Drug treatment of leucocytozoonosis has had limited success. No effective treatment has been found for *L. simondi*. Pyrimethamine (1 ppm) and sulfadimethoxine (10 ppm) administered simultaneously reportedly will prevent, but not cure, infections of *L. caulleryi*. Halofuginone has been used for treatment in Asia. Clopidol in feed effectively controlled *L. smithi* according to Siccardi *et al.* (98).

Control requires elimination of the insect vector from the environment of the vertebrate host. A large-scale aerial treatment program using an organophosphate insecticide (2% temephos granules) for control of larval *Simulium* substantially reduced adult and larval blackfly populations and reduced the level of *L. smithi* blood parasitemia in turkeys in one study (68).

Repellents sprayed within houses to discourage entrance of the insect vector lowered mortality and incidence of disease but did not completely prevent infection in the flock (33).

The control of *L. caulleryi* by vaccination using an oil-adjuvanted rR7 vaccine has shown promising results in both laboratory and field trials (55, 56). This vaccine uses a recombinant R7 protein (rR7) from second-generation *L. caulleryi* schizonts.

Avian Malaria

Parasites of the genus *Plasmodium* (phylum Apicomplexa) cause the presence of pigment in infected erythrocytes of the host. Schizogony occurs in blood, and gametocytes are found in mature erythrocytes. All species of *Plasmodium* are transmitted by mosquitoes. These characteristics distinguish them from *Haemoproteus* and *Leucocytozoon* species and other members of the family Plasmodiidae.

About 65 species of *Plasmodium* from more than 1000 different birds have been described, but only 35 are considered valid (9, 72). Species pathogenic for domestic fowl are found mostly in Asia, Africa, and South America. Malaria outbreaks have been recorded in North American birds of the orders Anseriformes, Passeriformes, and Columbiformes.

Etiology

Although many species of *Plasmodium* can be introduced into various domestic fowl, only a few appear to be natural parasites of these birds. *P. gallinaceum* occurs in jungle fowl and domestic hens; *P. juxtanucleare* parasitizes domestic hens and turkeys; *P. durae* and *P. griffithsi* occur in turkeys; *P. lophurae* of the fire-backed pheasant can also parasitize chickens and has been host-adapted to other domestic fowl and ducks; *P. fallax* of guinea fowl has been adapted to various domestic fowl; *P. hermani* will infect domestic and wild turkeys and bobwhite quail (31, 39).

Other species found primarily in passerine birds that can infect domestic fowl or have been experimentally transmitted to them include *P. relictum*, *P. elongatum*, *P. cathemerium*, and *P. circumflexum* (66).

Life Cycle

Only a general outline of the malarian life cycle can be given here. Consult Garnham (40) for information on the life cycles of various species. Greiner *et al.* (45) presented color plates of 24 species.

Avian plasmodia develop in culicine mosquitoes of the genera *Culex* and *Aedes*, and rarely in *Anopheles*. Gametocytes from an avian blood meal are taken up by the mosquito, after which gamete formation, oocyst development and sporogony occur. Infective sporozoites entering the avian host from the bite of a mosquito invade cells of the reticuloendothelial system and typically progress through two generations of primary exoerythrocytic schizonts: cryptozoites and metacryptozoites. Merozoites produced by the second generation are released into the bloodstream and invade erythrocytes. An interchange of parasites between blood and reticuloendothelial tissues may occur, resulting in secondary exoerythrocytic schizonts (phanerozoites) in many tissues, especially spleen, kidney, and liver endothelial cells. These may be responsible for subsequent heavy parasitemias.

The trophozoite inside an erythrocyte is known as the ring form because of its appearance. Romanowsky staining shows a band of blue cytoplasm surrounding a vacuole and a peripheral red-stained nucleus. The characteristic malarial pigment, visible in stained smears, is formed as the parasite consumes and metabolizes the host cell hemoglobin. Nuclear division (schizogony)

leads to formation of multiple nuclei, which bud off to form merozoites. The host cell ruptures to release merozoites for infection of other erythrocytes. After several asexual cycles, some merozoites differentiate into gametocytes and await ingestion by a suitable mosquito. The species of avian plasmodia vary in numbers of merozoites formed in exoerythrocytic and erythrocytic stages, in timing of the life cycle, and in morphology of various stages.

Pathology and Pathogenesis

The pathologic effects in avian hosts range from no apparent signs to severe anemia and death. *P. gallinaceum*, *P. juxtanucleare*, and *P. durae* are the most pathogenic for domestic fowl and may cause 90% mortality. Intense and severe anemia and generalized hypoxia may occur in acute *P. gallinaceum* malaria (66). A similar situation occurs in ducks affected with *P. lophurae*. Severe anemia may also occur in *P. juxtanucleare* infections.

Other pathologic changes occur in avian malaria. The exoerythrocytic stages of *P. gallinaceum* may block capillaries in the brain, resulting in death due to central nervous system dysfunction. Also, *P. durae* produces exoerythrocytic schizonts in the capillaries of the brain, sometimes producing high mortality (49).

Zoonotic Disease

Human malaria is an extremely important worldwide public health concern, however, no avian *Plasmodium* species has been reported to be capable of causing infection in humans (22).

Immunity

Immunologic factors, such as antigen-antibody complex and hemagglutinins, and such conditions as splenomegaly, anemia, and nephritis have been studied extensively in *P. gallinaceum* infections (82, 103).

Treatment and Control

The life cycle of the malaria parasite must be broken by the eradication of mosquitoes or by isolation of the flock from the vector by suitable housing. Although avian models have been used extensively in chemotherapeutic studies, information on potential preventative medications or treatments is limited. Studies with *P. durae* suggest that halofuginone is a possible preventive, and a combination of sulfachloropyrazine and sulfamonomethoxine could be used in treatment (49). Penguins are extremely susceptible to *Plasmodium* infection and have been treated successfully with a combined treatment of chloroquine and primaquine phosphate (43).

Haemoproteus Infections

Haemoproteus infections are characterized by schizogony (merogony) in visceral endothelial cells, gametocyte development in circulating erythrocytes, and the presence of pigment in granules in infected erythrocytes. Transmission is by various biting dipterans of the families Hippoboscidae and Ceratopogonidae (58). Characteristics of *Haemoproteus* are similar enough to *Plasmodium* and *Leucocytozoon* that the genera are placed in the same family, Plasmodiidae. Infections occur throughout tropical and temperate areas of the New and Old Worlds wherever vector species and avian hosts coexist.

There are about 128 species of *Haemoproteus* that have been reported from birds, mostly in wild waterfowl, raptors, passerines, and some other families of birds (72, 75). In surveys of wild birds, *Haemoproteus* species are the most commonly identified blood parasite. Many of the reported species become synonymous as life cycles are defined and cross-transmission studies are conducted, as shown by Bennett and coworkers (8, 116).

Species found in domestic poultry and pet birds include *Haemoproteus meleagridis*, in domestic and wild turkeys (44); *H. columbae* and *H. saccharovi* in pigeons and doves; and *H. nettionis* in waterfowl (78).

Etiology

H. columbae of pigeons and doves is the most extensively studied of these parasites. Sporogony occurs in 2 families of flies. Sporogony is completed in 6–7 days in the ceratopogonids or in 7–14 days in the hippoboscids. Schizonts (meronts) of various sizes and numbers of merozoites occur in the pulmonary vascular endothelium in alveolar septa of pigeons. The merozoites invade erythrocytes and mature into gametocytes (3). Further development requires ingestion by a suitable vector (arthropod) in a blood meal.

Vectors include the hippoboscid *Pseudolynchia canariensis* for *H. columbae* and the ceratopogonid *Culicoides* for *H. nettionis* (70). Vectors for *H. meleagridis* include *C. edeni*, *C. hinmani*, *C. arboricoli*, *C. knowltoni*, and *C. haemoproteus* (5).

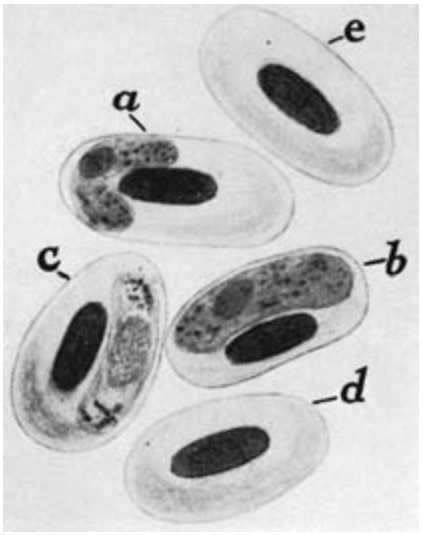
Atkinson (5) studied experimental infections of *H. meleagridis* in turkeys and partially defined the life cycle. He observed ookinetes, oocysts, sporozoites, and megaloschizonts, with at least 2 generations of schizogony. First-generation schizonts matured 5 and 8 days postinfection (PI) and produced elongate merozoites. Second-generation megaloschizonts developed after 8 and 17 days in cardiac and skeletal muscles and yielded spherical merozoites that developed into erythrocytic gametocytes (Fig. 28.18).

Pathogenesis and Pathology

Signs from experimental infections with *H. meleagridis* in turkeys include severe lameness, diarrhea, severe depression, emaciation, and anorexia (5). Anemia and enlarged livers occasionally are attributed to infections. Necropsy of wild turkeys revealed myopathy associated with megaloschizonts. Skeletal muscles contained numerous fusiform cysts oriented in parallel order with muscle fibers (6). Pigeons infected with *H. saccharovi* had enlarged gizzards. Muscovy ducks (*Carina moschata*) infected with *H. nettionis* suffered lameness, dyspnea, and sudden death with hemorrhage on the heart as well as edematous lungs and swollen firm livers, spleens, and kidneys, although this species was nonpathogenic for other species of ducks (64).

Diagnosis

The diagnosis of *Haemoproteus* infection requires microscopic examination of stained blood smears. Both restriction enzyme based methods and PCR asays have been developed which can



28.18. *Haemoproteus columbae*. Pigeon blood; (a, b) Macrogametocyte in erythrocyte. (c) Microgametocyte. (d, e) Normal erythrocyte. (Drake and Jones)

distinguish between *Haemoproteus* spp., *Plasmodium* spp., and *Leucocytozoon* spp. (7, 46).

Treatment and Control

Control of insect vectors may be of use in local situations (66). However, complete life cycles are not known for most species, precluding specific control recommendations. No drugs are approved for commercial use, although atebrin and plasmochin have marginal effects against *H. columbae* in experimental treatment

Trypanosomiasis

Although trypanosomes have been reported from many species of wild and domestic birds, their pathologic significance appears to be minimal or nil. Even the taxonomic grouping of these organisms is unclear.

Several species have been named, including *Trypanosoma avium*, *T. numidae*, *T. calmetti*, and *T. gallinarum*. The possibility that the latter 3 are synonyms of *T. avium* cannot be ruled out in absence of a rigorous taxonomic study (32). The ATCC lists two trypanosomes from avian species, *T. avium* and *T. bennetti* (72).

T. avium and its life cycle were described by Molyneaux (87), who summarized vector relationships for all avian trypanosomes and listed culicine mosquitoes and simuliids as known vectors.

Sarcocystosis

Sarcocystosis, previously called sarcosporidiosis, is caused by apicomplexan protozoa of the genus *Sarcocystis* Lankester 1882. The disease is recognized by the presence of elongated cysts (sarcocysts) located in muscles. The nature of the causative organism was unclear until discovery that coccidian oocysts are shed by the final host after eating flesh from another animal containing sarcocysts. These parasites are closely related to the *Eimeria* and other apicomplexans.

Sarcocystosis is not economically important to the poultry industry, but it occurs extensively in wild ducks and other birds. Many infected game birds are discarded by hunters for aesthetic reasons. Sarcocystosis from birds does not appear to be a public health hazard. The parasites are killed by cooking and storage at subfreezing temperatures. Mild signs were reported, however, by infected human volunteers given cysts from mammalian sources (72).

The apicomplexan nature of *Sarcocystis*, a controversial subject for the past century, and the historic aspects of sarcocystosis were reviewed by Spindler (104), Levine (73), Long (77), and Melhorn and Heydorn (83). Odening (93) provided a review of the taxonomy of *Sarcocystis* species, and Tenter (112) evaluated molecular methods used to clarify the taxonomy of this genus.

Incidence and Distribution

Avian sarcocystosis is found throughout the world in individual birds but is rare in domestic chickens. A study in northwest Ethiopia found that 6.6% of the chickens examined had sarcocysts within skeletal muscle specimens (118) but this high incidence of infection does not occur in modern poultry production systems. Two isolated cases of sarcocystosis in wild turkeys were reported in the southeastern United States (29, 111). The incidence is as high as 40% in ducks and 93% in grackles (34), and is influenced by species, age, and geographic location of the host. Sarcocystosis occurs more often in puddling than in diving ducks.

Etiology

Sarcocystis horvathi (*S. gallinarum*, *S. horvathi*) is the etiologic agent in chickens (74) and *S. rileyi* (*Balbiani rileyi*, *S. anatina*) in ducks. *Sarcocystis falcatula* has been described in numerous passerine, psittacine, and columbid species (74). Based on microscopic differences in the wall substance of microcysts, however, at least 5 different species of *Sarcocystis* are present in birds (25). These are two-host parasites, with a mammalian host serving as the definitive host.

Sarcocystis is classified in the phylum Apicomplexa, suborder Eimeriorina (74) and the family Sarcocystidae (72), and is characterized as multiplying by endodyogeny. Cysts or pseudocysts containing zoites are formed in parenteral cells of the host. It appears to have strong host specificity (73). The classification of *Sarcocystis* is based on discovery of its coccidial nature with a disporocystid (a tetrazoic isosporanlike oocyst), an obligatory two-host life cycle (often carnivore-herbivore), reproduction by endodyogeny, and characteristic ultrastructure (80, 100).

Morphology

Sarcocysts (third-generation meronts) of *S. rileyi*, also called Miescher's tubule, are elongate, with their long axis parallel to the muscle fibers (Fig. 28.19). They are whitish and smooth walled and appear cylindroid or spindle shaped when removed from the musculature. They are 1.0–6.5 × 0.48–1.0 mm (104). They have double-layered walls, an inner spongy fibrous layer,



28.19. Sarcocytosis in wild mallard; a severe, naturally occurring infection. (U.S. Dept Interior)

and an outer dense limiting membrane (73). Sarcocysts are divided into compartments, each of which contains numerous banana-shaped cystozoites (bradyzoites), also called Rainey's corpuscles. Cystozoites are 8–15 μm in length and 2–3 μm in width. Other developmental stages of *S. rileyi* are less well defined. The ultrastructure of *Sarcocystis* was described by Melhorn and Heydorn (83).

Life Cycle

Obligatory two-host life cycles have been described from 86 species of *Sarcocystis* (93). Two vertebrate hosts are required in the life cycle of all these species, usually a carnivorous predator or scavenger and the prey or food animal. Sexual reproduction occurs in the predator (definitive host) and asexual reproduction in the prey (intermediate host). The intermediate host becomes infected by fecal contamination from an infected definitive host.

Sarcocystis from shoveler ducks (*Anas dypeata*) were transmitted to the striped skunk (*Mephitis mephitis*) (20, 117). When muscle containing sarcocysts was eaten by skunks, sporocysts (1.4 × 12.4 μm) were shed sporadically from 19–63 days PI. Shoveler ducks orally administered sporocysts developed microcysts (80 × 16 μm) in skeletal muscle 85 days later and macrocysts (1–3 × <1 mm) 154 days PI (20). The opossum was susceptible to infection with tissue cysts from ducks (31). In another study (101), the transmission of *S. falcatula* was demonstrated with the opossum (*Didelphis virginiana*) serving as the definitive host. Although asexual parasites were not found in ducks (*Anas platyrhynchos*) given fecal sporocysts from opossums, the intermediate host spectrum of some avian species of *Sarcocystis* is apparently quite broad (16). Sarcocysts from grackles and cowbirds are also infective to the opossum.

Levine (74) lists the chicken as the intermediate host and the dog as the definitive host for *S. horvathi*. The life cycle for *S. horvathi* is not completely defined.

The life cycle of *Sarcocystis* spp. is summarized as follows: In cardiac, smooth, or skeletal muscle tissues are eaten by a definitive host, releasing cystozoites that penetrate the intestinal wall and develop into macrogametocytes and microgametocytes in subepithelial tissues. Oocysts (containing 2 sporocysts, each with four sporozoites) are produced and are shed in feces as fully sporulated sporocysts. Sporozoites are released when sporocysts are ingested by the intermediate host, and invade the mucosa of the intestine. Schizogony (merogony) occurs in endothelial cells of various organs. After several asexual generations, the merozoites develop into young cyst stages, containing metrocytes and later cystozoites, and mature into the third-generation meronts (sarcocysts) in myocardial, skeletal, and smooth muscle tissues (80, 101).

Pathogenicity

The pathogenicity of sarcocystosis in birds is variable. The presence of sarcocysts in the skeletal muscle and heart of waterfowl is often an incidental finding, with no clinical disease being evident. In contrast some avian species develop serious or fatal sarcocystosis as the protozoa undergo schizogeny in the endothelium of the infected intermediate avian host (94). Isolated cases of sarcocystosis reportly cause severe debility and death in wild turkeys and backyard chickens (29, 90, 111). Similar fatal disease has commonly been reported in psittacine birds where pulmonary hemorrhage and edema are the cause of death (94). Box and Duszynski (14) attributed death of 4 of 12 sparrows and morbidity of 3 of 6 canaries to experimental sarcocystosis.

Pathogenesis and Epidemiology

Naturally occurring and experimental sarcocystosis has been reported in 59 species and 11 orders of birds, including domestic ducks, chickens, and wild turkeys (13, 29, 104).

Transmission, Carriers, and Vectors

Attempts at direct transmission of *S. rileyi* to young ducks by oral, intramuscular (IM), and intravenous (IV) administration of cystozoites and cohabitation with infected ducks were unsuccessful, emphasizing the requirement of a second host (105).

Sporocyst-contaminated food is the common source of infection for the intermediate host (birds); infection in the carnivorous

definitive host (mammal) results from ingestion of sarcocyst-infected tissues of the intermediate hosts. Cystozoites from sarcocysts in confined migratory ducks were found to be viable for 3 years. Thus, intermediate hosts may serve as an available source of infection for prolonged periods over a widespread area. Sarcocytosis appears to be most prevalent in hosts that frequently drink from shallow or stagnant water (puddling ducks, cattle, sheep, or swine) (104).

Incubation Period

Infections are seldom found in juvenile grackles (34) or in juvenile ducks, suggesting a long incubation period. Microcysts and macrocysts were found in ducks 85 and 154 days PI, respectively (20), and macrocysts were observed in sparrows and canaries 70 days PI (15).

Clinical Signs

Sarcocysts usually are found in the skeletal muscles of birds. Spindler (104) reported that very heavy infections may cause signs of disease, and ducks may fly low and slowly. Adverse signs were not observed in experimentally infected ducks (20). Box and Duszynski (14) noted labored breathing and morbidity in canaries and sudden death in sparrows given oocysts. Psittacine birds with acute sarcocystosis likewise develop marked respiratory signs prior to death (94). Chickens with *Sarcocystis*-associated encephalitis exhibit distinct neurologic signs (90).

Gross Lesions

Sarcocysts running lengthwise in the musculature of the breast, thigh, neck, or esophagus are the usual lesions associated with avian sarcocytosis. Lung consolidation and splenomegaly were observed in infected canaries (14), and pulmonary edema and hemorrhage, hepatomegaly, and splenomegaly have been described in psittacine birds (90, 94). Lesions have not been seen in definitive hosts with experimental infections.

Histopathology

Fatty degeneration of muscles, enlargement and rupture of parasitized muscle fibers, and inflammatory responses around sarcocysts in muscles were reported (104). *Sarcocystis*-associated encephalitis has been described in chickens, turkeys, and other nondomesticated avian species (29, 90, 111). Systemic sarcocystosis has been described in a wild turkey with inflammatory changes associated with protozoal schizonts and merozoites in the heart, lung, and liver (29).

Immunity

Active and passive immunity have not been demonstrated. Animals have been immunized against sarcocystosis by repeated injections of untreated or formalin-treated toxin. Serum from immunized animals gives protection to other animals against the toxin (104).

Zoonotic Disease

While human cases of sarcocystosis do occur, they are typically asymptomatic and have been exclusively associated with the consumption of raw or undercooked beef or pork. (1).

Diagnosis

Diagnosis is based on the identification of sarcocysts or cystozoites in tissues. Large sarcocysts are seen easily in gross specimens; smaller cysts and cystozoites can be identified by histologic examination of muscle tissue. *Sarcocystis* schizonts and merozoites can be differentiated from other systemic protozoal infections (*Toxoplasma* and *Neospora*) by immunohistochemistry (81, 90). Molecular techniques using PCR amplification and restriction endonuclease digestion are also be used in the identification of *Sarcocystis* species (28). Infections of *Sarcocystis* may be diagnosed in the definitive host by identification of sporocysts in feces.

Serology

Sarcocystis reacts with cytoplasm-modifying antibody in the Sabin-Feldman dye test but cross-reacts with *Toxoplasma* (104). An indirect fluorescent antibody test in which cystozoites were used as the antigen was successful (114). Munday and Corbould (89) devised a complement-fixation test using an antigen prepared from sarcocysts and found that a titer of 1:10 was indicative of sarcosporidial infections. Serologic reactions have not been applied extensively in the diagnosis of sarcocystosis in birds.

Prevention and Control

Chemotherapy of avian sarcocystosis has no practical application at this time. The lack of chemotherapeutic or biologic control agents places the burden of control on prevention by breaking the infection cycle. Modern poultry production systems prevent the occurrence of sarcocystosis as the avian intermediate host is not exposed to the oocyst-contaminated excreta of the definitive host.

Toxoplasmosis

Toxoplasmosis is a parasitic disorder of mammals, birds, and reptiles affecting primarily the central nervous system but sometimes also the reproductive system, skeletal muscles, and visceral organs. The majority of infections are inapparent or latent, with overt toxoplasmosis resulting at times of stress or immunosuppression.

Only sporadic cases of toxoplasmosis in chickens and turkeys have been reported (26, 41, 95, 99). Studies of avian tissues using the mouse inoculation test and histologic examination indicate that a somewhat higher incidence of infection exists than is apparent based on the observation of clinical disease. Nevertheless, the disorder is uncommon in chickens and is of little significance to the health of commercial poultry.

Toxoplasmosis is a significant zoonotic disease and a human health problem of increasing importance due to a growing immunodeficient population (104). Humans are typically exposed to the disease by the ingestion of oocysts shed by felids, congenital infection, or the consumption of raw or undercooked meat products (113). Serologic prevalence of *Toxoplasma* infection in humans typically ranges between 10% and 80% and varies markedly based on environmental conditions and cultural dietary habits (113). An average seroprevalence of 14% was reported in

the United States (37). A multi-country case-control study of the source of *Toxoplasma* infection in pregnant women revealed that the greatest risk factors were ingestion of undercooked meats, soil contact, and travel outside of Europe and North America (21). Poultry products were not implicated as a significant risk factor in this study.

The true nature of *Toxoplasma* as close relatives of *Eimeria* was not known until 1969. The literature presents extensive overviews of history and current knowledge of toxoplasmosis (26, 37, 58, 62, 73, 113).

Etiology

A single species, *Toxoplasma gondii*, is the cause of toxoplasmosis in all hosts. Synonyms for the agent in avian hosts are *T. avium* and *T. paddae*.

T. gondii is a coccidian with sexual stages similar to *Isospora* (50). Endodyogeny, however, is unique to *T. gondii*. *Toxoplasma* is classified in the family Sarcocystidae, along with *Sarcocystis*, in the suborder Eimeriorina and phylum Apicomplexa (72).

Numerous *Toxoplasma* isolates have been designated strains based on differing pathogenicity in different hosts rather than on immunologic variation, although the latter may occur with some strains.

Free *T. gondii* zoites are crescent shaped (4–6 × 2–3 μm), with one extremity more rounded than the other and a nucleus near the rounded end. No pseudopods, cilia, or flagella are present. Ultrastructure of developmental stages was reviewed by Levine (73) and Ferguson *et al.* (38).

Life Cycle

Both schizogonic and gametogenic developmental cycles are known to occur in the intestinal epithelium of some members of the cat family (Felidae). Both an "enteroepithelial" cycle and an "extraintestinal" cycle have been described.

The enteroepithelial cycle occurs only in cats, resulting from infection by encysted organisms (bradyzoites), free or intracellular individual organisms (tachyzoites), or oocysts (35). The prepatent period is 24 days or longer if oocysts are ingested, 5–10 days after ingestion of tachyzoites, and only 3–5 days if bradyzoites are the source of infection. Asexual development (schizogony) occurs in the intestinal epithelium.

The sexual phase also occurs only in intestinal epithelial cells of Felidae. Gametocytes develop throughout the small intestine but more commonly in the ileum. Microgametocytes (7–10 × 5–8 μm) give rise to 12–32 microgametes (2–5 μm). Following fertilization of the macrogamete (13 μm), oocysts develop and detach unsporulated from the intestinal epithelium. Oocysts are shed for 7–20 days. Sporulation is complete in 1–5 days, depending on the environmental temperature and oxygen and results in the development of 2 sporocysts (6–8 × 5–7 μm), each containing 4 sporozoites.

In birds and other nonfelines, only the extraintestinal (tissue) cycle of *T. gondii* is known. After ingestion, *T. gondii* tachyzoites reproduce by endodyogeny within parasitophorous vacuoles of many cell types. Tachyzoites may spread to the brain, eye, heart, liver, lungs, and nucleated red blood cells of birds. Eight or more tachyzoites are produced in a host cell. A final generation of tachyzoites develops into tissue cysts, in which bradyzoites multiply by endodyogeny (113). Encysted bradyzoites develop intracellularly in the brain, heart, eyes, and skeletal muscles but are walled off as immunity develops. Cysts may persist for the life of the host or, if immunity wanes, bradyzoites may be released and a proliferation of tachyzoites renewed. The tissue cycle may reverse again and cysts form from tachyzoites (59, 62, 113).

Pathogenesis and Epidemiology

Infective oocyts of *T. gondii* are produced only by members of the Felidae (domestic cats, ocelots, pumas, jaguarundi, bobcats, and Asian leopards) (62). More than 63 species of birds and 27 species of other animals become infected from ingestion of oocysts and develop cysts in tissues without passing oocysts in the feces (99). Naturally occurring infections have been diagnosed in the chickens, turkeys, ducks, and many wild birds (17, 41, 78, 95). Ruiz and Frenkel (96) isolated *T. gondii* from 54% of chickens that were reared in small backyard poultry flocks in Costa Rica. In contrast, Kuticic (71) found that only 0.4 % of commercial chickens cultured positive for *T. gondii* in Croatia. Presumably, commercial chickens have little exposure to *T. gondii* oocysts, in contrast with free-range birds.

Transmission, Carriers, and Vectors

T. gondii has been reisolated from Japanese quail, bluejays, crows, turkeys, and chickens after experimental infections (27, 85). Tachyzoites and bradyzoites may be spread to birds by carnivorous ingestion, and sporulated oocysts are spread by cat feces.

The question of congenital infection occurring in chicks from naturally infected parents remains unresolved. Jacobs and Melton (60) found that 12 of 62 pools of reproductive tract tissues from chickens were infected with *T. gondii*, but the parasite could not be isolated from any of 108 eggs from these hens. In another study, 1 of 327 eggs from hens with chronic toxoplasmosis was positive. Iannuzzi and Renieri (52) concluded that toxoplasmas did not survive in unembryonated eggs and was not a factor in transmission. However, Caballero-Servin (18) reported successful transovarian transmission of the parasite by experimentally infecting hens, which resulted in embryonic mortality and congenital malformation of 18% of the surviving chicks.

Coprophagous arthropods such as flies and cockroaches can serve as transport hosts for the *Toxoplasma* (115). Earthworms ingest *Toxoplasma* oocysts and are a source of infection for chickens (96).

Course of the Disease

During the 1950s, several cases were reported that involved 12–50% of the chickens within a flock (12, 26). Clinical signs in affected chickens included anorexia, weight loss, pale combs, spasms, paralysis, and a loss of eyesight (26). In a recent case report, chickens affected with *T. gondii*-induced peripheral neuritis were emaciated and had difficulty standing (41). A wild turkey with systemic toxoplasmosis was emaciated, weak, and readily captured (95).

Susceptibility of chickens to *T. gondii* may vary with age of host, strain of infective agent, and route of infection. Oral inoculation with 10^3 or 10^5 *Toxoplasma* oocysts (30, 65) produced no clinical signs in 4-week-old chickens. Further, chickens inoculated with tachyzoites by intravenous or oral route showed no clinical disease or adverse production effects (65). Turkeys were also resistant to the oral inoculation of *Toxoplasma* oocysts (27).

Inoculation of chicks with tissue cysts by abnormal routes such as intracerebral (IC)(12) or intraperitoneal (IP) routes (67) produced clinical signs. Inoculation with tissue cysts IV, IP, IM, and SC also produced parasitemia and chronic infections in older birds. Clinical signs in experimentally inoculated chickens include anorexia, emaciation, paleness and shrinking of the comb, drop in egg production, whitish feces, diarrhea, incoordination, ataxia, trembling, opisthotonos, torticollis, blindness, and high mortality (11, 12, 67, 78).

Gross Lesions

Gross lesions include enlargement of liver and spleen, necrotic hepatitis, pericarditis, myocarditis, ulcerative enteritis, lung congestion, and encephalitis (12, 78).

Histopathology

In chickens inoculated by IC and IM routes, *Toxoplasma* tissue cysts were found in the cerebrum, brain stem, optic chiasma, and most frequently, around ventricles and in molecular and Purkinje layers of the cerebellum. Free trophozoites were seldom found, and then only in the brain. *Toxoplasma* cysts were found in the myocardium, pancreas, and testes of chickens infected intramuscularly (12).

Coagulation necrosis and diffuse sinusoidal congestion were observed in the liver. The myocardium, pancreas, and testes were diffusely infiltrated with lymphocytes, plasma cells, and heterophils. In the brain, infection caused lymphocytic lesions and plasma cell-cuffing of blood vessels; lymphocytic infiltration of choroid villi; ependymal proliferation of the lateral ventricle; thickening of leptomeninges; and gliosis of the lateral ventricle and around vessels of the cerebrum, brain stem, and cerebellum (12).

Oral inoculation of chickens with *Toxoplasma* oocysts produced focal necroses in the spleen, liver, and intestine and a mixed leukocyte infiltration (30). Lymphocytic foci were also noted in the myocardium, skeletal muscle and liver. Of the 12 chickens inoculated, only one developed lesions in the brain with perivascular leukocyte infiltration, gliosis, and one tissue cyst noted in the cerebrum (30).

Zoonotic Disease

Toxoplasmosis can affect numerous mammalian hosts, including humans, following the consumtion of oocytes shed by infected felids or uncooked meat containing infective *T. gondii* bradyzoites (1). Prevention of infection is by the sanitary disposal of cat feces and the thorough cooking of meat. Most postnatal infections are inapparent while congenitally acquired toxoplasmosis causes both severe disease and sequelae (1).

Diagnosis

T. gondii may be isolated and identified by injecting suspensions of infected tissues into various species of laboratory animals, chicken embryos, or cell cultures. Inoculation of mice IP or IC with suspensions of brain and heart are preferred methods of isolation (26). Mice inoculated with virulent strains die within a few days. Less virulent isolates may not produce mortality, and can only be detected serologically, or by examination for cysts 8–10 weeks after inoculation.

Impression smears of peritoneal fluids or tissues stained with Giemsa or tissue sections of brain, liver, spleen, lung, lymph nodes, and eye often suffice for direct microscopic observation of *Toxoplasma*.

Toxoplasma can be grown in the chorioallantoic cavity of 6–12-day-old embryonated chicken eggs. Embryos succumb 7–10 days PI with hemorrhage and nodular lesions in skin and viscera. Numerous yellow-white plaques 0.5–3.0 mm in diameter develop on the chorioallantoic and amniotic membranes. Smears of the chorioallantoic membrane and yolk sac stained with Wright's stain reveal numerous free and intracellular toxoplasmas.

Toxoplasma must be differentiated from other protozoa such as *Sarcocystis* and *Neospora*. Immunohistochemical stains capable of distinguishing between these species are available in several laboratories (29, 81, 90). A reliable PCR technique has been developed to detect *T. gondii* in formalin-fixed, paraffin-embedded tissue (51). The use of PCR testing in diagnosis of toxoplasmosis in fresh tissues was reviewed by Morgan (88).

Serology

Historically, avian toxoplasmosis serology testing was considered impractical because most avian species do not seroconvert using the human dye test. However, the modified agglutination test and ELISA test detected antibodies within 2 weeks of inoculation of chickens, and at 68 days post-inoculation (30). The latex agglutination test was judged insensitive in detecting *T. gondii* antibodies, and no antibodies were detected by the dye test or the indirect hemagglutination test. Other workers have used an ELISA test to demonstrate seroconversion in chickens and pigeons within 2 or 3 weeks of inoculation, respectively (11).

Treatment, Prevention, and Control

Chemotherapy has not been used to control avian toxoplasmosis. Prevention of avian toxoplasmosis requires management practices that eliminate the source of infective tachyzoites and oocysts by preventing exposure to rodents, coprophagous arthropods, and especially cats. Oocysts disseminated throughout the premises are resistant to common laboratory detergents, acids, and alkalis and are, therefore, difficult to destroy. However, they may be destroyed by ammonia, drying, and a temperature of 55°C (73).

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Chapter 29

Nutritional Diseases

Kirk C. Klasing

Poultry require the presence of at least 36 nutrients in their diet in appropriate concentrations and balance (Table 29.1). Commonly available feedstuffs are usually deficient in many nutrients, and diets must be supplemented with the deficient nutrients, usually from purified sources. Errors in the formulation or milling may sometimes result in deficient or toxic levels of one or more nutrients. Severely deficient or toxic levels often are expressed as characteristic pathologies to organs and tissues. Marginally inadequate supplies often result in suboptimal growth, impaired resistance to infectious diseases, decreased egg production, or lowered hatchability. Frequently, it is the task of the veterinarian to determine whether an ailment is nutritional in its origin or whether nutrition is a contributing factor to a specific clinical problem. It may be difficult to recognize a partial nutritional deficiency because nonspecific signs may be brought about by a number of causes, including infectious diseases and toxicants.

The quantitative nutrient requirements of the young growing chick and turkey and for light breeds of laying hens are quite well established (Table 29.1); however, the requirements of growing chicks and poults after the first few weeks of age and the requirements of male and female broiler and turkey breeding fowls for many nutrients have not been determined experimentally.

Food substances of importance in nutrition of poultry are water, proteins and amino acids, carbohydrates, fats, vitamins, and essential inorganic elements.

Water

Water holds a unique position in nutrition mainly due to its physical properties. Because of its solvent and polar properties, it acts as a transport medium for other nutrients and products of metabolism and enhances cell reactions. Because of its high specific heat, it can absorb the heat of reactions produced in the oxidation of carbohydrates and fats with little rise in temperature. Water evaporates readily, removing many calories from the body as latent heat of vaporization. These and many other functions explain why the animal can exist much longer without food than without water.

Unlike larger farm animals, chickens and turkeys must have access to a continuous water supply, because they drink only small amounts at a time. An insufficient amount results in decreased growth, egg production, and resistance to heat stress.

The quantity of water consumed by chicks is correlated directly with the salt content of their diet (5, 179). Sodium and potassium in the form of bicarbonate salts cause similar increases in water intake of broiler chicks. Chlorine and phosphorus also increase water intake, but not as much as sodium or potassium and calcium has little effect (12, 179). Excess dietary protein and deficiencies of amino acids result in increased water intake (12). The effect of protein is presumably due to increased excretion of nitrogen and minerals such as phosphorus and sulfur that are constituents of protein.

Proteins and Amino Acids

Commercial diets usually are formulated using a “least cost” approach, and meeting the protein and amino acid requirements impacts greatly the cost of the diet. For this reason, the limiting amino acids in the diet are typically supplied with very little margin of safety. The protein requirement represents the collective need for 10 absolutely essential amino acids (arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine), 2 amino acids (cysteine and tyrosine) that can be synthesized from essential amino acids, 2 amino acids that are essential for the young chick (glycine or serine and proline), plus additional amino acids to satisfy the nitrogen requirement for synthesis of nonessential amino acids, purines, pyrimidines, and other nitrogenous compounds.

Practical ingredients usually are limiting in one or more amino acids. Grains such as corn, milo, and wheat are most deficient in lysine; whereas soybean meal is most deficient in methionine. It is often cost effective to supply these limiting amino acids in the form of synthetic amino acids, especially lysine and methionine. Other amino acids such as threonine, tryptophan, arginine, and isoleucine can become limiting when unusual protein sources are used or when the dietary protein level is reduced. Diets that are devoid of animal byproducts are often fortified heavily with feed grade amino acids.

In contrast to the specific signs that may occur as a result of vitamin or mineral deficiencies, the effects of essential amino acid deficiencies are nonspecific: reduced growth, reduced feed consumption, decreased egg production and egg size, and loss of body weight in adults. The decrease in feed intake occurs within hours of consumption of a deficient diet and is due to a distortion in plasma and tissue amino acid levels. Marginal amino acid deficiencies often result in increased food intake or the maintenance of food intake, with concomitant reduction of body weight gain and lean tissue growth resulting in increased body fat. Severe deficiencies also result in altered body composition.

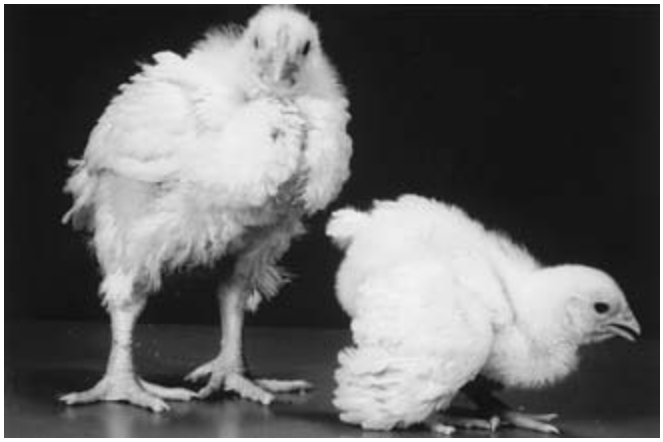
Table 29.1. Nutrient requirements of poultry.[1]

Nutrient	Unit	Required Level			
		Egg-laying strain from 0–6 wk	White-egg layers at 100 % production	Broiler from 0–3 wk	Turkey from 0–4 wk
Crude protein	%	18.00	15.00	23.00	28.00
Arginine	%	1.00	0.70	1.25	1.60
Glycine + serine	%	0.70	–	1.25	1.00
Histidine	%	0.26	0.17	0.35	0.58
Isoleucine	%	0.60	0.65	0.80	1.10
Leucine	%	1.10	0.82	1.20	1.90
Lysine	%	0.85	0.69	1.10	1.60
Methionine	%	0.30	0.30	0.50	0.55
Methionine + cystine	%	0.62	0.58	0.90	1.05
Phenylalanine	%	0.54	0.47	0.72	1.00
Phenylalanine + tyrosine	%	1.00	0.83	1.34	1.80
Threonine	%	0.68	0.47	0.80	1.00
Trytophan	%	0.17	0.16	0.20	0.26
Valine	%	0.62	0.70	0.90	1.20
Linoleic acid	%	1.00	1.00	1.00	1.00
Calcium	%	0.90	3.25	1.00	1.20
Nonphytate phosphorus	%	0.4	0.25	0.45	0.60
Potassium	%	0.25	0.15	0.30	0.70
Sodium	%	0.15	0.15	0.20	0.17
Chlorine	%	0.15	0.13	0.20	0.15
Magnesium	mg/kg	600	500	600	500
Manganese	mg/kg	60.00	20.00	60	60.00
Zinc	mg/kg	40.00	35.00	40.00	70.00
Iron	mg/kg	80.00	45.00	80.00	80.00
Copper	mg/kg	5.00	?	8.00	8.00
Iodine	mg/kg	0.35	0.035	0.35	0.40
Selenium	mg/kg	0.15	0.06	0.15	0.20
A	IU/kg	1500	3000	1500	5000
D	IU/kg	200	300.00	200.0	1100
E	IU/kg	10.00	5.00	10.00	12.00
K	mg/kg	0.50	0.50	0.50	1.75
Riboflavin	mg/kg	3.60	2.50	3.60	4.00
Pantothenic acid	mg/kg	10.00	2.00	10.00	10.00
Niacin	mg/kg	27.00	10.00	35.00	60.00
B_{12}	mg/kg	0.009	0.004	0.01	0.003
C	mg/kg	0	0	0	0
Choline	mg/kg	1300	1050	1300	1600
Biotin	mg/kg	0.15	0.10	0.15	0.25
Folic acid	mg/kg	0.55	0.25	0.55	1.00
Thiamin	mg/kg	1.00	0.70	1.80	2.00
Pyridoxine	mg/kg	3.00	2.50	3.50	4.50

[1]Requirements are taken from NRC (1994) and are based on diets with standard energy contents and a dry matter of 90%.

Some amino acids have additional effects. Methionine deficiency may exacerbate choline or vitamin B_{12} deficiencies owing to its role in methyl group metabolism. Lysine deficiency causes impaired pigmentation of bronze turkey poults, the biochemical basis of which is unknown (75), and can result in stunting and retarded development in chicks (Fig. 29.1). Arginine deficiency tends to cause the wing feathers to curl upward, giving the chick a distinct ruffled appearance. Several other amino acids have been reported to affect feather growth and structure (75, 159).

When animals are provided with dietary protein in excess of their requirements, the surplus protein is catabolized, and the nitrogen released is converted to uric acid. A large excess of protein may cause hyperuricemia and exacerbate articular urate deposition ("articular gout") in birds that are genetically susceptible



29.1. Lysine deficiency. Stunting and retarded development are apparent in this chick (right) fed a diet without sufficient lysine when compared with the normal control chick (left) fed adequate lysine. (Swayne)

(15, 178). Excesses of individual amino acids due to feed mixing errors are especially toxic. Methionine is the most toxic of the amino acids and also the amino acid most likely to supplemented to a poultry diet. The relative order of toxicity of amino acids for growing chickens fed a corn and soybean diet is methionine > phenylalanine > tryptophan > histidine > lysine > tyrosine > threonine > isoleucine > arginine > valine > leucine (59, 80). Acute toxicity of individual amino acids manifests as a severe decrease in food intake and usually can be diagnosed by high levels of the toxic amino acid in the blood. Oxidation of excess methionine results in the release of sulfate, which generates two moles of acid. Oxidation of phosphorylated amino acids and dibasic amino acids also contributes to metabolic acidosis. Thus, high levels of dietary protein or of methionine cause metabolic acidosis and may contribute to a variety of problems in poultry, including poor bone mineralization, thinning of eggshells, and poor growth.

Carbohydrates

This food component is the primary source of metabolizable energy in practical poultry diets. Starch and sucrose are used readily by the chick. Intestinal lactase activity is low in chickens; this limits the amount of lactose that can be tolerated. Milk byproducts, such as whey, are excellent sources of B vitamins, and although beneficial at low levels, excessive levels in the diet cause growth depression and severe diarrhea. The latter condition, characteristic of lactose intolerance in many species, is caused by influx of water into the lower digestive tract and by microbial fermentation of undigested lactose.

Fats

Fats are important in the diet of poultry as concentrated sources of energy and sources of the essential nutrient linoleic acid. Linoleic acid cannot be synthesized but can be converted to arachidonic acid by poultry. Both fatty acids are important constituents of cell organelles, membranes, and adipose tissue and have additional physiologic roles as precursors of prostaglandins. Lack of these fatty acids in the diet of young chicks results in suboptimal growth and enlarged fatty livers (90). Essential fatty acid deficiency in laying hens results in lowered egg production, egg size, and hatchability (133).

Reduced concentrations of arachidonic acid and increased concentrations of eicosatrienoic acid in tissue and egg lipids are a characteristic sign of essential fatty acid deficiency.

Unsaturated fatty acids may undergo oxidative rancidity, with multiple effects: Essential fatty acids are destroyed; aldehydes that are formed may react with free amino groups in proteins, reducing amino acid availability; and the active peroxides generated during rancidification may destroy activities of vitamins A, D, and E and water-soluble vitamins such as biotin. Producers of vitamin A supplements have enhanced the stability of this vitamin by mechanical means, wherein minute droplets of vitamin A are enveloped in a stable fat, gelatin, or wax, forming a small bead that prevents most of the vitamin from coming into contact with oxygen until it is digested in the intestinal tract. The addition of synthetic antioxidants to poultry feeds provides further protection of vitamin A and other essential nutrients.

Vitamins

The term vitamin refers to a heterogeneous group of fat-soluble and water-soluble chemical compounds essential in nutrition that bear no structural or necessary functional relationship to each other. All recognized vitamins with the exception of vitamin C are dietary essentials for poultry. Although amounts of various vitamins needed in poultry diets range from parts per million to parts per billion, each is required for normal metabolism and health.

A marked deficiency of a single vitamin in the diet of a chick or poult results in failure of the metabolic process in which that particular vitamin is concerned. This causes a vitamin-deficiency disease, which in some instances exhibits characteristic macroscopic or microscopic changes. In several instances, a single disease may result from a deficiency of any one of several nutrients. Chondrodystrophy ("perosis"), for example, occurs in young chicks or poults when the diet is deficient in manganese or any one of the following vitamins: choline, nicotinic acid, pyridoxine, biotin, or folic acid. Chondrodystrophy is an anatomic deformity of leg bones of young chickens, turkeys, pheasants, and other birds, which is characterized by decreased linear bone growth, enlargement of the tibiometatarsal joint, twisting or bending of the distal end of the tibia and proximal end of the metatarsus, with secondary varus or valgus deformation of the legs, and, finally, slipping of the gastrocnemius tendon from its condyles. This last lesion causes complete crippling in the affected leg; if both legs are affected, death usually results because the chick or poult cannot secure food and water. Analysis of the diet may be the only way to determine whether a specific nutritional deficiency is responsible for the condition.

Vitamins A and D and riboflavin are most likely to be deficient

if special attention is not given to provide them when feed is formulated. Because of extraction and purification of many common ingredients, however, and the tendency to omit animal proteins and high-fiber ingredients such as alfalfa meal and wheat mill by-products from diets, amounts of several other vitamins have decreased to sometimes deficient levels. These are vitamins E, B_{12}, and K; pantothenic acid, nicotinic acid, biotin, and choline. Poultry rations usually are formulated to contain more than adequate amounts of all vitamins, providing margins of safety to compensate for possible losses during feed processing, transportation, and storage, and variations in feed composition and environmental conditions.

Vitamin A

Vitamin A is essential in poultry diets for growth, optimal vision, and integrity of mucous membranes. Because epithelial linings of alimentary, urinary, genital, and respiratory systems are composed of mucous membranes, these are the tissues in which lesions of vitamin A deficiency are most readily observed. Vitamin A aldehyde, or retinal, is a component of visual pigments in sensory cells of the retina within which cis-trans isomerization of the isoprenoid side chain plays an essential role in the detection of light. Vitamin A, as retinoic acid, functions in morphogenesis during embryonic development, the maintenance of epithelial tissues, mucus production, bone growth, immunity, and a variety of other essential processes. Most dietary vitamin A is in the form of retinol and retinal, which are oxidized by cells to retinoic acid. Retinoic acid mediates the effects of vitamin A by regulating gene expression.

Vitamin A Deficiency

Clinical Signs and Signalment. When adult chickens or turkeys are fed a diet severely deficient in vitamin A, signs and lesions usually develop within 2–5 months, depending on the amount stored in liver and other tissues of the body. As deficiency progresses, chickens become emaciated and weak, and their feathers are ruffled. Egg production decreases sharply; the length of time between clutches increases; and hatchability is decreased. A watery discharge from the nostrils and eyes is noted, and eyelids are often stuck together. As the deficiency continues, milky white, caseous material accumulates in the eyes. At this stage of the disease, eyes fill with this white exudate to such an extent that it is impossible for the chicken to see unless the mass is removed; in many cases, the eye is destroyed. Most signs in adult turkeys are similar to those in chickens (16, 88).

Marginal deficiencies of vitamin A may cause epithelial damage in the oropharynx and esophagus without accompanying growth depression (16). More severe deficiencies result in keratinization of intestinal enterocytes, a decrease in the number of goblet cells, decreased alkaline phosphatase activity, decreased expression of brush-border enzymes, and blunting of villi. Impaired growth rate of deficient broiler chicks appears to be secondary to diminished digestive function (200). The incidence and severity of blood spots in eggs of chickens is increased in vitamin A deficiency. The amount of vitamin A required to minimize blood spot incidence may be slightly higher than the requirement for good production and health of the laying hens (87, 154). Vitamin A deficiency also results in abnormal embryonic development (201).

Vitamin A-deficiency signs in chicks and poults are cessation of growth, drowsiness, weakness, incoordination, emaciation, and ruffled plumage. If deficiency is severe, they may show ataxia not unlike that of vitamin E deficiency (87), although the 2 conditions can be differentiated by histologic examination of the brain (3). Periorbital edema may occur (Fig. 29.2A). In acute vitamin A deficiency, lacrimation usually occurs, and a caseous material may be seen under the eyelids. Xerophthalmia may not be observed because in acute deficiency, chicks often die of other causes before the eyes become affected. Increased testes weight, spermatogenesis, and comb development may occur in young cockerels marginally deficient in vitamin A (140). Vitamin A-deficient cocks have decreased sperm counts, reduced sperm motility, and a high incidence of abnormal sperm (148).

Pathology. Vitamin A-deficiency lesions first appear in the pharynx and are confined largely to mucous glands and their ducts. The original epithelium is replaced by a keratinizing epithelium (i.e. squamous metaplasia) that blocks ducts of the mucous glands, causing them to become distended with secretions and necrotic materials. Squamous metaplasia can be found in nasal mucosa (Fig. 29.2B). Small white nodules are found in the nasal passages, mouth, esophagus, and pharynx and may extend into the crop. Nodules range in size from microscopic lesions to 2 mm in diameter (Fig. 29.2C). As the deficiency progresses, lesions enlarge, are raised above the surface of the mucous membrane, and have a depression in the center. Small ulcers surrounded by inflammatory products may appear at the site of these lesions. This condition resembles certain stages of fowl pox, and the 2 conditions can be differentiated only by microscopic examination. Bacterial and viral infections often occur because of breakdown of the mucous membrane.

Clinical signs and lesions of vitamin A deficiency of the respiratory tract are variable; it is difficult to differentiate this condition from infectious coryza, fowl pox, and infectious bronchitis. In vitamin A deficiency, thin diphtheritic membranes and nasal plugs usually are limited to the cleft palate and its adjacent epithelium. They may be removed easily without bleeding. Atrophy and degeneration of the respiratory mucous membrane and its glands occur. Later, the original epithelium is replaced by a stratified squamous keratinizing epithelium. In early stages of vitamin A deficiency in chickens, turbinates are filled with seromucoid water-clear masses that may be forced out of the nodules and cleft palate by application of slight pressure. The vestibule becomes plugged and overflows into paranasal sinuses. Exudate may also fill sinuses and other nasal cavities, causing swelling of one or both sides of the face. Mucous membranes, cleared of inflammatory products, appear thin, rough, and dry.

Similar lesions frequently may be found in the trachea and bronchi. In early stages, these may be difficult to see. As the condition progresses, the mucous membrane is covered with a dry, dull fine film that is slightly uneven, whereas a normal membrane is even and moist. In some cases, small nodule-like parti-

cles may be found in or beneath the mucous membrane in the upper part of the trachea.

Chronic vitamin A deficiency causes damage to the kidney tubules, which leads to azotemia and visceral urate deposits (e.g., "visceral gout") in severe cases (178).

Histopathology. The first histologic lesion of vitamin A deficiency is atrophy and deciliation of columnar-ciliated epithelium of the respiratory tract (174). Nuclei often present with marked karyorrhexis. A pseudomembrane formed by the atrophying and degenerating ciliated cells may hang as tufts on the basement membrane; later these are sloughed. During this process, new cylindric or polygonal cells may be formed singly or in pairs and appear as islands beneath the epithelium. These new cells proliferate, and their nuclei enlarge, containing less chromatin as they develop. Cell boundaries are less clearly defined; finally, the columnar ciliated epithelial lining of nasal cavities and communicating sinuses, trachea, bronchi, and submucous glands are transformed into a stratified squamous keratinizing epithelium. Lesions in glands of tongue, palate, and esophagus (Fig. 29.2D) are similar to those of the respiratory tract (175).

Histopathologic examination of tissues from nasal passages of chicks serves as a sensitive indicator of borderline deficiencies of vitamin A (101). Chicks receiving suboptimal levels show lesions that resemble in basic character, but not in severity, those described by Seifried (174) for complete deficiency of vitamin A.

According to Wolbach and Hegsted (221, 222), vitamin A deficiency in young chicks and ducks causes marked retardation and suppression of endochondral bone growth. The proliferating zone is reduced. Hypertrophied cells accumulate, surrounded by uncalcified matrix. Vascular invasion of the epiphyseal cartilage is reduced and exhibits irregular patterns such as branching. The number of endosteal and periosteal osteoblasts is decreased, leading to impaired bone growth and thinning of bone cortex. Bone remodeling is inhibited. Disproportionate growth of brain and spinal cord relative to that of the axial skeleton appears to cause compression of brain tissue. Increased cerebrospinal fluid pressure is one of the earliest signs of vitamin A deficiency (224).

An increased frequency of atretic ovarian follicles containing hemorrhages either throughout the follicle or between the theca interna and granulosa cell layer has been observed in chickens exposed to vitamin A deficiency over a period of 5–8 months (25). Vitamin A deficiency has been reported to decrease hatchability of chicken and turkey eggs and to increase mortality of chicks and poults that do hatch (10, 87). Thompson *et al.* (195) produced a severe vitamin A deficiency in developing embryos by supplementing breeder diets with retinoic acid. This form of vitamin A permits egg production but does not support embryonic development. Embryos die—always in the same stage of development. The complete trunk and head are formed, and the head is rotated slightly to one side. No differentiation of major blood vessels occurs, and an expanded area of vasculosa is seen forming a "blood ring" at the sinus terminalis.

Treatment of Deficiency. Poultry found to be severely deficient in vitamin A should be given a stabilized vitamin A preparation at a level of approximately 10,000 IU vitamin A/kg of ration. Absorption of vitamin A is rapid; therefore, chickens or turkeys not in advanced stages of deficiency should respond promptly, except for blindness, which may be permanent.

Hypervitaminosis A

Baker *et al.* (20) reported that the administration of 200 mg retinyl acetate per kg of body weight per day to growing chickens adversely affects skeletal development. Chicks have lighter and shortened tibiae exhibiting widened epiphyseal growth plates with irregular tunneling by blood vessels. Widening results from increased numbers of hyperplastic chondrocytes. Bones exhibit reduced osteoblastic activity and increased bone and blood alkaline phosphatase activity. Ventricular dilation and brain swelling are also observed.

Tang *et al.* (191) administered 330 or 660 IU vitamin A per kg body weight per day to commercial broilers. Chicks had an unsteady gait and were reluctant to walk within a few days of treatment with excess vitamin A. They became anorexic by 9 days and developed conjunctivitis, adhesions of the eyelids, and encrustations around the mouth. Tibiae had widened epiphyseal growth plates due primarily to accumulation of hypertrophic chondrocytes. These investigators reported other abnormalities of the tibia including hyperosteoidosis and metaphyseal sclerosis. Frontal bones of the skull were thinner and more porous and exhibited thickened osteoid seams.

Signs of hypervitaminosis A in leghorn chicks differed from those of broiler chicks administered similar levels of vitamin A (191). The epiphyseal growth plates in tibiae from leghorn chicks were normal in width but contained a narrower proliferative or maturation zone and a wider hypertrophic zone. Osteoid seams were normal. Leghorns had normal parathyroid morphology, whereas parathyroid hyperplasia was observed in broiler chicks.

It must be noted that the reported histopathology of hypervitaminosis A is not entirely consistent among laboratories. The nature of the cell population contributing to widening of the epiphyseal growth plate differed in the preceding studies (20, 191). Wolbach and Hegstead (220, 223), moreover, reported that vitamin A excess caused narrowing of the growth plate in their early studies involving young chicks and ducks.

Vitamin D

Vitamin D is required by poultry for proper metabolism of calcium and phosphorus in the formation of normal skeleton, hard beaks and claws, and strong eggshells. It functions in the regulation of calcium metabolism by stimulating the intestinal absorption of calcium, influencing osteoblast and osteoclast activity, and increasing renal tubular reabsorption of calcium in response to metabolic demands for calcium.

Vitamin D can be synthesized from 7-dehydrocholesterol in the skin under the influence of ultraviolet light. Although this synthesis can reduce the dietary requirement for vitamin D to some extent (62), it is not sufficient to satisfy the requirements of fowl under normal conditions of poultry and egg production. Poultry diets commonly are supplemented with cholecalciferol (vitamin D_3); however, 25-hydroxycholecalciferol is also some-

times used and has a slightly higher bioavailability when fed at low levels (11). The plant source of vitamin D activity, ergosterol (vitamin D_2), is not efficiently used by poultry (41) and is not usually used as a supplement.

The metabolically active form of vitamin D is formed by 2 enzymatic hydroxylations of cholecalciferol (vitamin D_3), the first yielding 25-hydroxycholecalciferol in the liver, and the second yielding 1,25-dihydroxycholecalciferol in the kidneys (8, 55). The second hydroxylation is tightly regulated by calcium status, being activated by low blood calcium, phosphate or parathyroid hormone. 1,25-dihydroxycholecalciferol is much more potent in promoting calcium absorption and bone mobilization than its precursors, vitamin D_3 and 25-hydroxycholecalciferol. Many other hydroxylation products of 25-hydroxycholecalciferol have been identified. In particular, 24R,25-dihydroxyvitamin D_3 appears to be an essential vitamin D_3 metabolite for both normal bone integrity and healing of fracture in chicks (176).

Vitamin D Deficiency

Clinical Signs and Signalment. In confined laying hens, signs of deficiency begin to occur as soon as 2 weeks after they are deprived of vitamin D. The first sign is a marked increase in the number of thin-shelled and soft-shelled eggs, followed soon after by marked decrease in egg production. Biochemical indicators include a rapid decrease in the concentrations of 25-hydroxycholecalciferol and 1,25-dihydroxycholecalciferol in the blood, followed soon thereafter by a decrease in blood calcium concentration (176, 197, 199). Egg production and eggshell strength may vary in a cyclic manner. Several cycles of decreased egg production and shell strength may each be followed by periods of relatively normal production and shell strength.

Individual hens may show temporary loss of the use of the legs, with recovery after laying an egg that is usually shell-less. During periods of extreme leg weakness, hens show a characteristic posture that has been described as a "penguin-type squat." Later, beak, claws, and keel become very soft and pliable. The sternum usually is bent, and ribs lose their normal rigidity and turn inward at the junction of the sternal and vertebral portions, producing a characteristic inward curve of the ribs along the sides of the thorax.

Vitamin D metabolism has been implicated in problems of eggshell quality. Soares *et al.* (180) reported that 2 strains of chickens that had been selected for divergence in eggshell strength and thickness differed in their blood concentrations of 1,25-dihydroxycholecalciferol: The strain having higher eggshell quality also had significantly higher concentrations of the vitamin D metabolite. When hens of a commercial strain of leghorns received 30 μg of vitamin D_3 or 5 mg of 1-α-hydroxycholecalciferol (a putative synthetic precursor of 1,25-dihydroxycholecalciferol), the latter resulted in greater tibial calcium and phosphorus content, tibial breaking strength, and eggshell mineralization. Bar *et al.* (21) reported that the inclusion of 2 or 5 μg/kg of 1,25-dihydroxycholecalciferol in the diet of aging hens increased shell weight and density in the first egg of the clutch and decreased the rate of decline of both measures in subsequent eggs of the clutch. Other studies (197, 198, 199) confirm that 1,25-dihydroxycholecalciferol supports egg production and is effective in promoting eggshell mineralization and minimizing eggshell breakage when it is used at a concentration of 5 μg/kg in the diet of leghorn hens.

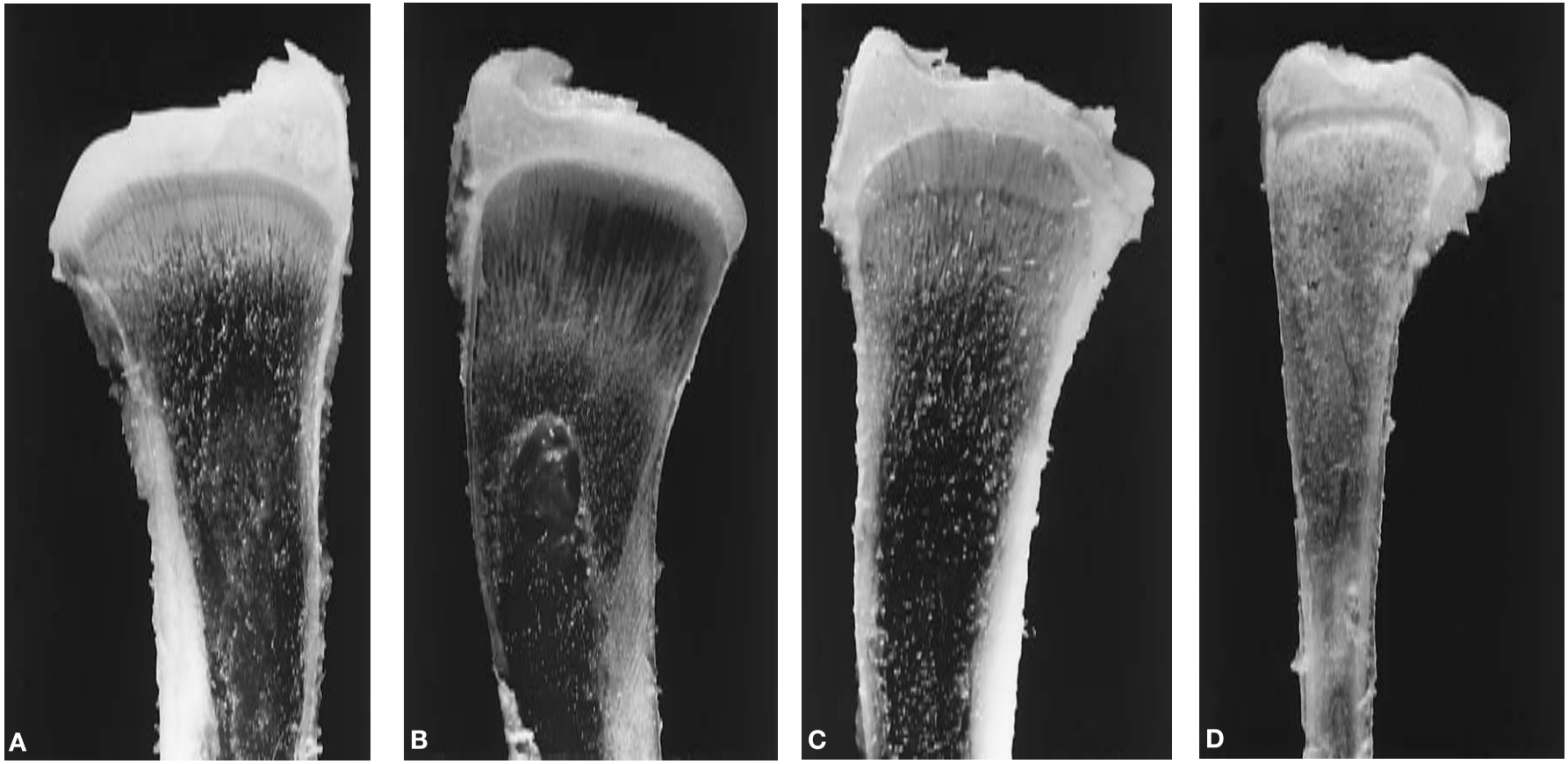
Hatchability is reduced markedly by vitamin D deficiency. Chicks and poults that do not hatch have a high incidence of chondrodystrophy in which the upper or lower mandible is shortened to the extent that occlusion of the mandibles is abnormal (183, 186). The synthetic vitamin D analogues 25-hydroxycholecalciferol, 1-α-hydroxycholecalciferol, and 1,25-dihydroxycholecalciferol support adequate egg production and eggshell strength, but only 25-hydroxycholecalciferol is effective in supporting hatchability (1, 8). Evidence strongly suggests that the other 2 analogues are poorly transported into the egg (8, 64, 181). Manley and coworkers (127) reported that the addition of 1100 ICU of 25-hydroxy-cholecalciferol to diets of turkey hens that already contained 2200 ICU of vitamin D_3 improved the hatchability of fertile eggs. This interesting observation appears at odds with other evidence that 900 IU of vitamin D_3 per kilogram of diet is adequate for hatchability of turkey eggs (183).

In addition to retarded growth, the first sign of vitamin D deficiency in chicks or poults is rickets, characterized by severe fragility and bending of long bones due to poor mineralization. Between 2 and 3 weeks of age, beaks and claws become soft and pliable, and birds walk with obvious effort and take a few unsteady steps before squatting on their hocks, which they rest upon while swaying slightly from side to side. Feathering is poor. A marked increase in serum phosphatase is perhaps the first indicator of a borderline rachitic condition.

Pathology. In laying and breeding chicken and turkey hens receiving deficient vitamin D, characteristic changes observed on necropsy are confined to bones and parathyroid glands. The latter become enlarged from hypertrophy and hyperplasia. Bones are soft and break easily. Well-defined knobs are present on the inner surface of the ribs at the costochondral junction (rachitic rosary) (Fig. 29.2E). Many ribs show evidence of pathologic fracture in this region. In chronic vitamin D deficiency, marked skeletal distortions become apparent. The spinal column may bend downward in the sacral and coccygeal region; the sternum usually shows a lateral bend and an acute dent near the middle of the breast. These changes reduce the size of the thorax with consequent crowding of vital organs. The beak may be soft and pliable (Fig. 29.2F).

The most characteristic internal signs of vitamin D deficiency in chicks and poults are a bending of the ribs at their juncture with the spinal column and a bending of the ribs downward and posteriorly (Fig. 29.2E). Poor calcification can be observed at the epiphysis of the tibia or femur (Fig. 29.3). Bones of vitamin D-deficient chicks have reduced calcium content with an increased proportion of osteoid, and a greater proportion of bone mineral is present as a low-density amorphous form of calcium phosphate (57).

Vitamin D deficiency results in widening of the epiphyseal plate, hypertrophy, and softening of bone. Enlargement of the epiphyseal plate initially is due to widening of the proliferating and hypertrophic zones; as the deficiency progresses, it may be



29.3. Effects of nutrient deficiencies on tibiotarsal bones of broiler chickens. (Swayne) A. Control fed an adequate diet. B. Phosphorus deficiency. Prominent wide zone of hypertrophy. C. Calcium/phosphorus deficiency. Widened zone of proliferation. D. Lysine deficiency. Hypoplasia.

primarily the former (83, 92, 123). Long and coworkers (123) noted that the hypertrophic zone exhibits irregular contours—wider in some areas and narrower in others—among and within affected birds. The widening of the proliferating zone appears to be the result of delayed chondrocyte hypertrophy rather than increased chondrocyte replication (109). As the deficiency progresses, the columns of chondrocytes in the degenerating hypertrophic zone of the epiphyseal plate become shortened and thickened and exhibit an irregular pattern of invasion by metaphyseal blood vessels. Irregular patterns of cartilage and bone development occur in the primary and secondary spongiosa (92, 123). Porosity of cortical bone, sometimes leading to fractures (Fig. 29.2G), increases due to resorption of bone in haversion canals. Fractures also may occur elsewhere (Fig. 29.2H). Decrease in trabecular bone volume due to increased osteoclast resorbing activity contributes to diminished mechanical strength of long bones (103). Increasing the dietary calcium level to levels twice those normally required maintains normal epiphyseal cartilage width and metaphyseal bone histomorphology and mineralization in chicks fed vitamin D deficient diets (103).

The histopathology of rickets differs significantly depending on the cause of the disease (109, 121, 122, 123). Refer to the section on calcium and phosphorus for further information on this topic.

Another skeletal disorder, tibial dyschondroplasia, frequently is observed in broiler chickens (refer to Chapter 31 for a description of the pathology). It has been produced experimentally by decreasing the ratio of calcium to phosphorus in the diet (60, 155) or by altering the ratio of these nutrients and increasing the dietary concentration of anions, such as chloride (79). This condition persists even when experimental diets contain generous levels of vitamin D_3 (212). The incidence and severity of tibial dyschondroplasia were reduced or prevented when the diets were supplemented with 1,25-dihydroxycholecalciferol (61, 63, 155), suggesting that the metabolic conversion of vitamin D_3 to 1,25-dihydroxycholecalciferol is not sufficient under some conditions to meet the need for this metabolite for normal bone development.

Commercial sources of 25-dihydroxycholecalciferol have become available, and this vitamin D metabolite has intermediate activity for prevention of tibial dyschondroplasia (209).

Treatment of Deficiency. Hooper *et al.* (89) found that feeding a single massive dose of 15,000 IU vitamin D_3 cured rachitic chicks more promptly than when generous levels of the vitamin were added to feed. This single oral dose protected cockerels against rickets for 8 weeks and pullet chicks for 5 weeks. In giving massive doses to rachitic chicks, it should be remembered that excess vitamin D can be harmful. The dose should be scaled to the degree of deficiency, and excessive amounts of vitamin D should not be added to feed.

Hypervitaminosis D

The relative toxicity of vitamin D and its metabolites follows the same pattern as their bioactivity: $D_2 < D_3 <$ 25-dihydroxycholecalciferol < 1,25-dihydroxycholecalciferol. Elevated rates of calcium absorption and mobilization from the bone cause elevated levels of calcium in body fluids, resulting in soft-tissue calcification, cellular degeneration, and inflammation. Vitamin D toxicity is exacerbated by high levels of dietary calcium or phosphorus, especially in the growing chick. In broiler chicks, pathology can be detected at 30,000 IU/kg of vitamin D_3 when fed throughout the growth period. Lesions include atrophy of parathyroid gland associated with the proliferation of connective tissues, Ca deposits in basal areas of the aortic valve and renal tubular lumina, and epithelial calcification in blood vessel walls that in the brain caused vacuolization and necrosis (42). Hens are generally more resistant to vitamin D toxicity than growing chicks, but toxic levels can be transferred to the egg causing excessive mobilization of eggshell calcium and late embryonic death. Very high levels of vitamin D_3—4 million IU or more/kg diet—rapidly induce renal damage from dystrophic calcification of kidney tubules. Calcification may be less often observed in the aorta and other arteries. A moderate excess of vitamin D has been reported to increase the incidence of eggshell pimpling (74). The latter appears to be due to excessive localized calcareous deposits on and within the eggshell that when scraped off the shell, often expose the underlying eggshell membranes. In laying hens, 25-dihydroxycholecalciferol becomes toxic at around 825 μg/kg feed (192).

Vitamin E

Vitamin E deficiency produces encephalomalacia, exudative diathesis, and nutritional myopathy (muscular dystrophy) in chicks; enlarged hocks and dystrophy of the ventricular musculature in turkeys; and nutritional myopathy in ducks. Vitamin E also is required for normal embryonic development in chickens, turkeys, and probably ducks.

In its alcoholic form, vitamin E is a very effective antioxidant. It is an important protector in feeds of the essential fatty acids and other highly unsaturated fatty acids as well as vitamins A and D_3, carotenes, and xanthophylls. Diets that contain high levels of unstabilized polyunsaturated fatty acids become depleted of vitamin E and are most likely to cause deficiencies. Selenium (Se) at dietary concentrations of 0.04–0.1 ppm has been shown to prevent or cure exudative diathesis in vitamin E-deficient chicks (170, 171, 172). Selenium at 0.1–0.2 ppm effectively prevents myopathies of ventriculus and heart in young poults (173).

Vitamin E plays multiple roles in poultry nutrition. It is required not only for normal reproduction but also as nature's most effective antioxidant for prevention of encephalomalacia, in a specific role interrelated with action of selenium for prevention of exudative diathesis and turkey myopathies, and in another role interrelated with selenium and cystine for the prevention of nutritional myopathy. Vitamin E has a low level of toxicity for poultry, and problems with excess levels are often due to induction of a deficiency in another fat-soluble vitamin—like vitamin A or K.

Clinical Signs, Signalment, and Pathology of Deficiency

No outward signs occur in mature chickens or turkeys receiving very low levels of vitamin E over prolonged periods. However, hatchability of eggs from vitamin E-deficient chickens or turkeys is reduced markedly (93). Embryos from hens fed rations low in vitamin E may die as early as the fourth day of incubation or con-

siderably later, depending on the severity of the deficiency. Turkey embryos may have bilateral cataracts that can cause blindness (66). Testicular degeneration occurs in males deprived of vitamin E for prolonged periods (4).

Encephalomalacia in Chicks. Encephalomalacia is a nervous syndrome characterized by ataxia or paresis (Fig. 29.4A), backward or downward retractions of the head (sometimes with lateral twisting), forced movements, decreasing coordination, rapid contraction and relaxation of the legs, and finally complete prostration and death. Even under these conditions, complete paralysis of wings or legs is not observed. The deficiency usually manifests itself between day 15 and 30 of the chick's life, although it has been known to occur as early as day 7 and as late as the day 56. Dietary long-chain polyunsaturated fatty acids, especially C18:2n6, increase the severity of encephalomalacia (70).

The cerebellum, striatal hemispheres, medulla oblongata, and mesencephalon are affected most commonly in the order named (147). In chicks killed soon after the appearance of signs of encephalomalacia, the cerebellum is softened and swollen, and the meninges are edematous (Fig. 29.4B). Minute hemorrhages are often visible on the surface of the cerebellum. The convolutions are flattened. As much as four-fifths of the cerebellum may be affected, or lesions may be so small they cannot be recognized grossly. A day or two after signs of encephalomalacia appear, necrotic areas present a green-yellow opaque appearance. One or two days later, the cerebellum may become pale and shrunken (Fig. 29.4C).

In the corpus striatum, necrotic tissue is frequently pale, swollen, and wet and in early stages becomes sharply delineated from remaining normal tissue. The greater portion of both hemispheres may be destroyed. In other cases, lesions are apparent only on microscopic examination. Medullary lesions are not so readily noted in a macroscopic examination.

Histologically, lesions include circulatory disturbances (ischemic necrosis), demyelination, and neuronal degeneration (Fig. 29.4D,E). Meningeal, cerebellar, and cerebral vessels are markedly hyperemic, and a severe edema usually develops. Capillary thrombosis often results in necrosis of varying extent. In the normal chick cerebellum, myelinated tracts exhibit a strongly positive reaction with Luxol fast blue; whereas in affected chicks, the staining reaction is markedly diminished, diffusely or locally accentuated. Degenerative neuronal changes occur everywhere but are most prominent in Purkinje cells and in large motor nuclei. Ischemic cell change is most frequently encountered. Cells are shrunken and intensely hyperchromatic, and the nucleus is typically triangular. Peripheral chromatolysis with the Nissl substance packed along the periphery of the cell nucleus is also common.

Signs of encephalomalacia in turkey poults are similar to those observed in chicks (97). Poults with paresis usually do not have brain lesions but have poliomyelomalacia (Fig. 30.4F).

Exudative Diathesis in Chicks. Exudative diathesis is an edema of subcutaneous tissues (Fig. 29.5) associated with abnormal permeability of capillary walls. In severe cases, chicks stand with their legs far apart as a result of accumulation of fluid under the ventral skin. This green-blue viscous fluid is seen easily through the skin, because it usually contains some blood components from slight hemorrhages that appear throughout the breast and leg musculature and in the intestinal walls. Distention of the pericardium and sudden deaths have been noted. Chicks suffering from exudative diathesis show a low ratio of albumin to globulins in blood (73).



29.5. Exudative diathesis in chicks.(Scott)

Onset of exudative diathesis coincides with appearance of peroxides in tissues. Plasma activities of selenium-dependent glutathione peroxidase decrease sharply (141). Intracellular and extracellular isozymes of glutathione peroxidase catalyzes the neutralization of hydrogen peroxide and lipoperoxides that can cause oxidative damage to structural elements of the cell, particularly membrane lipids. Noguchi *et al.* (141) proposed that vitamin E in the capillary membranes and the selenium-containing enzyme glutathione peroxidase of plasma protect the capillary membrane against oxidative damage. This may explain the dual role of vitamin E and selenium in the prevention of exudative diathesis and other vitamin E/selenium-responsive diseases (172, 189). Another selenium-dependent enzyme, phospholipid hydroperoxide glutathione peroxidase, probably also is involved in the protection of membranes from oxidative damage.

Nutritional Myopathy (Muscular Dystrophy) in Chickens, Ducks, and Turkeys. When vitamin E deficiency is accompanied by a sulfur amino acid deficiency, chicks show signs of nutritional myopathy—particularly of the breast muscle—at about 4 weeks of age. The condition is characterized by light-colored streaks of easily distinguished affected bundles of muscle fibers in the breast (Fig. 29.4G). A similar dystrophy occurs throughout all skeletal muscles of the body in vitamin E-deficient ducks.

The initial histologic change is hyaline degeneration. Mitochondria undergo swelling, coalesce, and form intracytoplasmic

globules. Later, muscle fibers are disrupted transversely. Extravasation separates groups of muscle fibers and individual fibers. The transuded plasma usually contains erythrocytes and heterophilic leukocytes. In more chronic conditions, reparative processes dominate the picture. There is a pronounced proliferation of cell nuclei and also fibroplasia, leaving a scar in the degenerate muscle.

Vitamin E and selenium deficiency in chickens and especially in turkeys may result in an extreme myopathy of the ventriculus (gizzard) (Fig. 29.4H) and heart muscles (173).

Enlarged Hock Disorder in Turkeys. Turkeys receiving diets low in vitamin E and also containing readily oxidizable fats or oils may develop characteristic hock enlargements and bowed legs at approximately 2–3 weeks of age (169). If poults are allowed to continue on these diets, hock enlargements usually disappear by the time the poults are 6 weeks of age, only to reappear in more severe form when they reach 14–16 weeks, especially in toms raised on wire or slat floors. Creatine excretion is increased, and muscle creatine levels are reduced. The need for vitamin E may be related to a protection of biotin that otherwise might be destroyed in the presence of rancidifying fats or oils.

Treatment of Deficiency

If not too far advanced, exudative diathesis and nutritional myopathy in chicks are readily reversed by the administration of proper levels of vitamin E and selenium by injection, by oral dosing, or in feed. Encephalomalacia may or may not respond to treatment with vitamin E, depending on the extent of damage to the cerebellum. Ventricular myopathy in turkeys is prevented by supplementing deficient diets with vitamin E or selenium. It is not affected by the dietary level of sulfur amino acids.

Vitamin K

Vitamin K is required for synthesis of prothrombin. It is a cofactor in the posttranslational carboxylation of glutamic acid in prothrombin, osteocalcin, and several other calcium binding proteins. The product, a-carboxyglutamic acid, is anionic at physiologic pH and functions in the binding of Ca to protein during blood clotting. In the absence of vitamin K, an abnormal prothrombin lacking α-carboxyglutamic acid is secreted into the blood by the liver (71). Because prothrombin is an important part of the blood-clotting mechanism, deficiency of vitamin K results in markedly prolonged blood-clotting time (95); an affected chick or poult may bleed to death from a slight bruise or other injury. Vitamin K deficiency reduces the α-carboxyglutamic acid content of bone in laying hens and growing chicks (111).

Clinical Signs, Signalment, and Pathology of Deficiency

Signs of vitamin K deficiency occur most frequently 2–3 weeks after chicks are placed on a vitamin K-deficient diet. Presence of sulfaquinoxaline in feed or drinking water may increase incidence and severity of the condition. Large hemorrhages appear on the breast, legs, and wings, and/or in the abdominal cavity. Chicks show an anemia that may result partly from loss of blood but also from development of a hypoplastic bone marrow.



29.6. Typical stargazing pose displayed by chick suffering from thiamin deficiency. (Scott)

Although blood-clotting time is a fairly good measure of vitamin K deficiency, a more accurate one is obtained by determining prothrombin time. Inadequate vitamin K in breeder diets causes increased embryo mortality late in incubation. Dead embryos appear hemorrhagic.

Treatment of Deficiency

Within 4–6 hours after vitamin K is administered to deficient chicks, blood clots normally, but recovery from anemia or disappearance of hemorrhages cannot be expected to take place promptly.

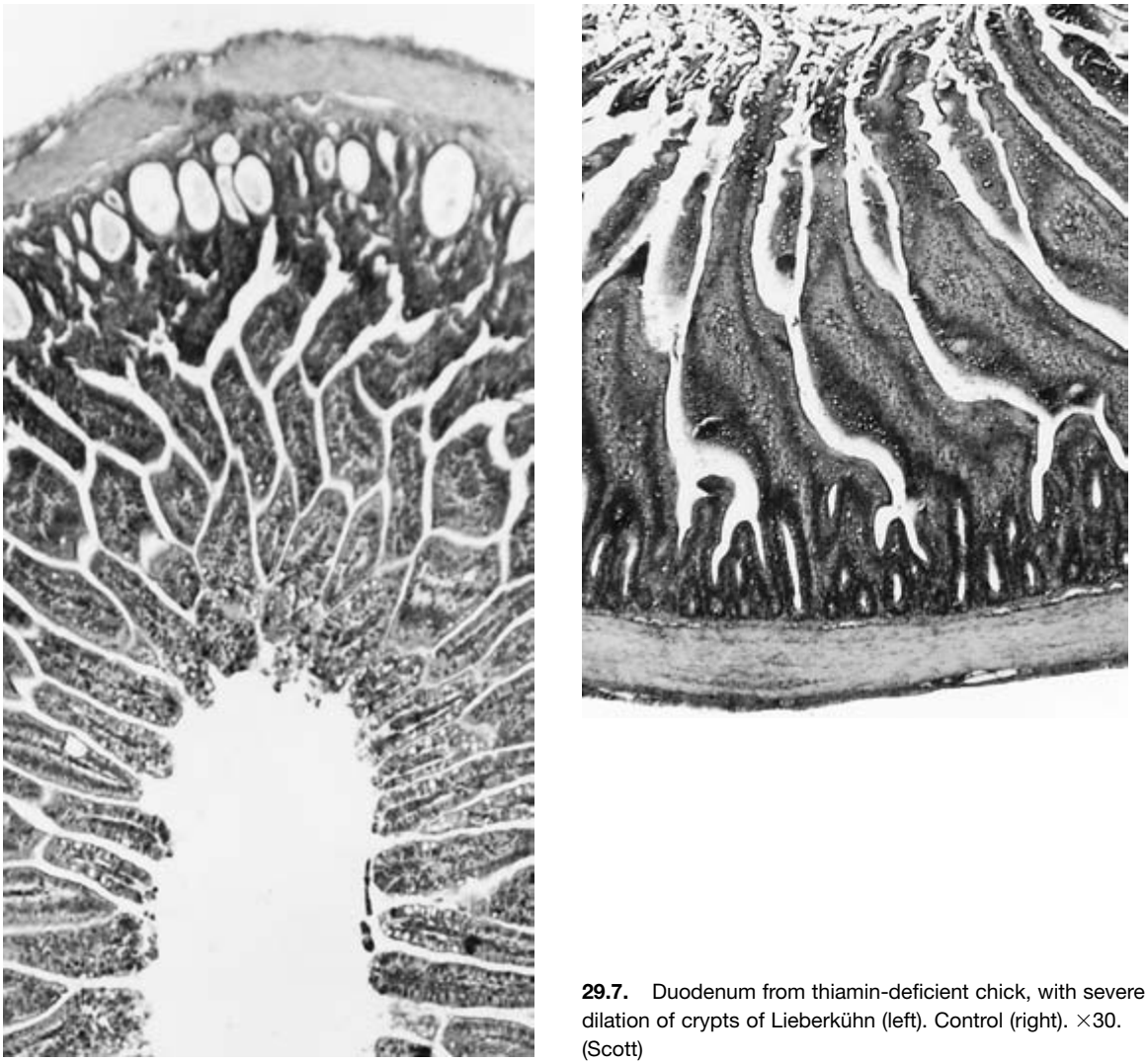
Thiamin (Vitamin B_1)

Thiamin is converted in the body to an active form, thiamin pyrophosphate, which is an important cofactor in oxidative decarboxylation reactions and aldehyde exchanges in carbohydrate metabolism. Deficiency of thiamin leads to extreme anorexia, polyneuritis, and death.

Clinical Signs, Signalment, and Pathology of Deficiency

Polyneuritis is observed in mature chickens approximately 3 weeks after they are placed on a thiamin-deficient diet. In young chicks, it may appear before 2 weeks of age. Onset is sudden in young chicks but more gradual in mature birds. Anorexia is followed by loss of weight, ruffled feathers, leg weakness, and an unsteady gait. Adult chickens often show a blue comb. As the deficiency progresses, apparent paralysis of muscles occurs, beginning with the flexors of the toes and progressing upward, affecting the extensor muscles of legs, wings, and neck. The chicken characteristically sits on its flexed legs and draws back the head in a "stargazing" position (Fig. 29.6). Retraction of the head is due to paralysis of the anterior muscles of the neck. The chicken soon loses the ability to stand or sit upright, and it topples to the floor, where it may lie with the head still retracted.

The body temperature may drop to as low as 35.6°C. A progressive decrease in respiration rate occurs. Adrenal glands hypertrophy more markedly in females than males. Apparently, the degree of hypertrophy determines the degree of edema, which occurs chiefly in the skin. The epinephrine content of the adrenal



29.7. Duodenum from thiamin-deficient chick, with severe dilation of crypts of Lieberkühn (left). Control (right). ×30. (Scott)

gland increases as the organ hypertrophies. Atrophy of genital organs is more pronounced in males than females. The heart shows a slight degree of atrophy; the right side may be dilated, the auricle being more frequently affected than the ventricle. Atrophy of the stomach and intestinal walls may be sufficiently severe to be easily noted.

Crypts of Lieberkühn in the duodenum of deficient chicks become dilated (Fig. 29.7) (78). Mitosis of epithelial cells in the crypts decreases markedly; in advanced stages of deficiency the mucosal lining disappears, leaving a connective tissue framework. Necrotic cells and cell debris accumulate in the enlarged crypts. Exocrine cells of the pancreas show cytoplasmic vacuolation with the formation of hyaline bodies.

Treatment of Deficiency

Chickens suffering from thiamin deficiency respond in a matter of a few hours to oral administration of the vitamin. Because thiamin deficiency causes extreme anorexia, supplementing feed with the vitamin is not a reliable treatment until after chickens have recovered from acute deficiency.

Riboflavin (Vitamin B_2)

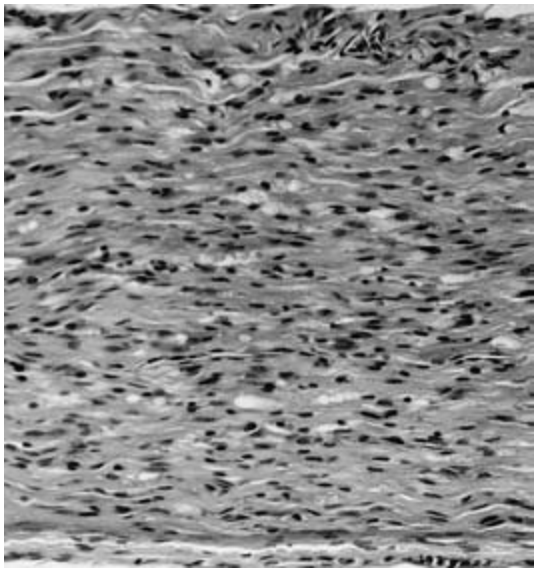
Riboflavin is a cofactor in many enzyme systems in the body. Examples of riboflavin-containing enzymes are NAD- and NADP-cytochrome reductases, succinic dehydrogenase, acyl dehydrogenase, diaphorase, xanthine oxidase, L- and D-amino acid oxidases, L-hydroxy acid oxidases and histaminase, some of which are vitally associated with oxidation-reduction reactions involved in cell respiration.

Clinical Signs, Signalment, and Pathology of Deficiency

When chicks are fed a diet deficient in riboflavin, they grow very slowly and become weak and emaciated; their appetite is fairly good; diarrhea develops between the first and second weeks. Chicks do not walk except when forced to, and then they frequently walk on their hocks with the aid of their wings. Leg paralysis may be more prevalent than curled-toe paralysis (46). Toes are curled inward when both walking and resting (Fig. 29.8). Chicks are usually found in a resting position. The wings often droop as though it were impossible to hold them in the normal position. Leg muscles are atrophied and flabby, and the skin



29.8. Curled-toe paralysis (riboflavin deficiency). Typical signs include poor growth, reluctance to stand or walk, sitting on hocks, and toes curled inward. (Swayne)



29.9. Curled-toe paralysis. Peripheral neuropathy characterized by axonal swelling and degeneration, Schwann cell activation and proliferation, and myelin degeneration. ×70. (Swayne, Barnes)

is dry and harsh. Young chicks in advanced stages of deficiency do not move around but lie with their legs sprawled out.

Riboflavin deficiency in young turkeys is characterized by poor growth, poor feathering, leg paralysis (165), and by encrustations in the corners of the mouth and on the eyelids. Severe dermatitis of the feet and shanks—marked by edematous swelling, desquamation, and deep fissures—appears in some deficient poults (131).

In severe cases of riboflavin deficiency, chicks show marked swelling and softening of sciatic and brachial nerves (35). Sciatic nerves usually undergo the most pronounced changes, sometimes reaching a diameter 4–5 times normal size. Histologic examination of affected nerves shows degenerative changes in myelin sheaths of the main peripheral nerve trunks (Fig. 29.9). This may be accompanied by axis cylinder swelling and fragmentation. Schwann cell proliferation, myelin changes, gliosis, and chromatolysis occur in the spinal cord. Fine structural examination of the sciatic nerve reveals that redundant folds and loops of myelin form symmetric or asymmetric expansions of the sheath resulting in segmental demyelination (35). In cases of curled-toe paralysis, degeneration of the neuromuscular end plate and muscle tissues is often found. Riboflavin is probably also essential for myelin metabolism of the main peripheral nerve trunks. No gross dystrophy develops, although muscle fibers are in some cases completely degenerated. The sciatic nerve exhibits myelin degeneration in one or more branches. Similar changes are apparent in the brachial nerve trunks. Chicks fed riboflavin-deficient diets develop pancreatic and duodenal lesions as described for thiamin deficiency in addition to the more classic nervous signs (78).

A deficiency of riboflavin in the diet of hens results in decreased egg production, increased embryonic mortality, and an increase in size and fat content of the liver. Hatchability of eggs decreases within 2 weeks after hens are fed a riboflavin-deficient diet but improves to near normal levels within 7 days after adequate amounts of riboflavin are added to the diet. Embryos that fail to hatch from eggs of hens fed diets low in this vitamin are dwarfed and show a high incidence of edema, degeneration of Wolffian bodies, and defective down. The down is referred to as "clubbed" and results from failure of the down feathers to rupture the sheaths, causing feathers to coil in a characteristic way.

Riboflavin is transported to the egg by riboflavin-binding protein (RfBP). Chickens that are genetically unable to produce RfBP lay eggs that support embryonic development until 13 or 14 days of incubation (208). However, they lack sufficient riboflavin to complete embryogenesis. At day 10 of incubation, embryos become severely hypoglycemic and begin to accumulate intermediates of fatty acid oxidation. The major metabolic consequence of riboflavin deficiency appears to be a severe impairment of fatty acid oxidation due to an 80% reduction in the activity of medium-chain acyl-CoA dehydrogenase. The nervous system of embryos that fail to hatch from eggs laid by hens fed riboflavin-deficient diets has degenerative changes very much like those described in riboflavin-deficient chicks (65).

Treatment of Deficiency

Two 100-μg doses of riboflavin should be sufficient for treatment of riboflavin-deficient chicks or poults, followed by incorporation of an adequate level in the ration. When the curled-toe deformity is of long standing, however, irreparable damage has occurred and administration of riboflavin no longer cures the condition.

Pantothenic Acid

Pantothenic acid is a component of coenzyme A, which is involved in the formation of citric acid in the Krebs cycle, synthesis and oxidation of fatty acids, oxidation of keto acids resulting

from deamination of amino acids, acetylation of choline, and many other reactions.

Clinical Signs, Signalment, and Pathology of Deficiency

Signs of pantothenic acid deficiency in chicks are difficult to differentiate from those of biotin deficiency; deficiencies of either result in dermatosis, broken feathers, chondrodystrophy, poor growth, and mortality. Pantothenic acid-deficient chicks are characterized by retarded and rough feather growth. Chicks are emaciated, and definite crusty scablike lesions appear in corners of the mouth. Eyelid margins are granular, and small scabs develop on them. Eyelids frequently are stuck together by a viscous exudate; they are contracted, and vision is restricted. There is slow sloughing of the keratinizing epithelium of the skin. Outer layers of skin between the toes and on bottoms of the feet sometimes peel off; small cracks and fissures appear at these points. These cracks and fissures enlarge and deepen, so chicks move about very little. In some cases, skin layers of the feet of deficient chicks cornify and wartlike protuberances develop on the balls of the feet.

Necropsy shows the presence of a pasty substance in the mouth and an opaque gray-white exudate in the proventriculus (157). The liver is hypertrophied and may vary in color from a faint to dirty yellow. The spleen is atrophied slightly. Kidneys are somewhat enlarged. Nerves and myelinated fibers of the spinal cord show myelin degeneration (153). These degenerating fibers occur in all segments of the cord down to the lumbar region.

Pantothenic acid is required in the diet of breeding hens for normal hatchability of eggs (72). Beer *et al.* (24) observed that the peak day of embryonic mortality depends on the degree of pantothenic acid deficiency and that borderline deficiencies produce extremely weak chicks that fail to survive unless injected immediately with pantothenic acid (200 μg intraperitoneally). Subcutaneous hemorrhage and severe edema are signs of pantothenic acid deficiency in the developing chicken embryo (24).

Pantothenic acid deficiency in chicks produces duodenal and pancreatic lesions as described under thiamin deficiency (but of lesser extent), dermatosis, and severe ataxia progressing to inability to stand. In addition, there is pronounced lymphocytic necrosis and lymphoid depletion in the bursa of Fabricius, thymus, and spleen (78).

Treatment of Deficiency

Pantothenic acid deficiency appears to be completely reversible, if not too far advanced, by oral treatment or injection with the vitamin followed by restoration of an adequate level in the diet.

Nicotinic Acid (Niacin)

Nicotinic acid is the vitamin component in 2 important coenzymes, nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP), that are extensively involved in carbohydrate, fat, and protein metabolism. They are especially important in metabolic reactions that furnish energy. One or both coenzymes take part in the anaerobic and aerobic oxidation of glucose, glycerol synthesis and catabolism, fatty acid synthesis and oxidation, and oxidation of acetyl coenzyme A via the Krebs cycle.

Niacin-Tryptophan-Pyridoxine Interrelationships

Tryptophan pyrrolase catalyzes the initial reaction in the major metabolic pathway of tryptophan catabolism. Picolinic carboxylase regulates an important branch point in the pathway at which an intermediate either enters a sequence of reactions resulting in its degradation to carbon dioxide, water, and ammonia or enters a biosynthetic pathway leading to NAD synthesis. Picolinic carboxylase catalyzes the first reaction in the degradative pathway, whereas the first reaction in the NAD pathway occurs nonenzymatically. High picolinic carboxylase activity limits the synthesis of NAD from tryptophan.

Key enzymes in the metabolism of tryptophan require vitamin B6 as a cofactor and limit the overall pathway in vitamin B6 deficiency. Briggs *et al.* (30, 31) first showed that niacin requirements of chicks and hens depend on the level of tryptophan in the diet. When tryptophan is marginally adequate, chickens can synthesize approximately 1 mg of niacin from 45 mg of dietary tryptophan (18, 39, 58). Ducks, in contrast, are much less efficient: approximately 1 mg niacin can be synthesized from 180 mg of dietary tryptophan (40). This difference in efficiency of conversion of tryptophan to niacin is reflected in a markedly higher niacin requirement for ducks than chicks. It has been attributed to relatively high picolinic carboxylase activity in ducks (40, 58).

Clinical Signs, Signalment, and Pathology of Deficiency

The main lesion of nicotinic acid deficiency in young chicks, turkeys, and ducks is an enlargement of the hock joint (chondrodystrophy) and bowing of the legs (39, 58). The main difference between this condition and the chondrodystrophy of manganese or choline deficiency is that in nicotinic acid deficiency, the Achilles tendon rarely slips from its condyles. Scott (169) showed that both nicotinic acid and vitamin E are required for prevention of the disorder in turkeys. Briggs (30) described further signs of nicotinic acid deficiency as inflammation of the mouth, diarrhea, and poor feathering. Hock disorders and lesions of the mouth are prominent lesions in ducks and chicks, respectively (39). Niacin/tryptophan deficiency in chicks produces duodenal and pancreatic lesions comparable to those of thiamin deficiency (78).

Ringrose *et al.* (158) observed reduced feed consumption and body weight, decreased rate of egg production, and reduced hatchability of eggs when hens were fed a semipurified diet based on casein and gelatin as the sources of protein and lacking in supplemental niacin. No signs of pathology were observed. Although no evidence has been obtained of any need to supplement practical diets of mature chickens (2) with nicotinic acid, niacin supplementation was reported to increase egg size with turkey breeders (82).

Treatment of Deficiency

Supplementing a deficient ration with required amounts of nicotinic acid has little or no effect on cases that have progressed to the extent that the tendon has slipped from its condyles (chondrodystrophy) or on advanced cases of enlarged hock disorder in adult tom turkeys. Excessive supplementation should be avoided because levels above 0.75% dietary niacin cause decreased bone thickness dimensions and bone strength (96).

Pyridoxine (Vitamin B_6)

Pyridoxine is required in several enzymes, particularly those involved in transamination and decarboxylation of amino acids. The coenzymes are pyridoxal phosphate and pyridoxamine phosphate.

Clinical Signs, Signalment, and Pathology of Deficiency

Severely pyridoxine-deficient chicks show depressed appetite, poor growth, chondrodystrophy, and characteristic nervous signs. Chicks show jerky, nervous movements of the legs when walking and often undergo extreme spasmodic convulsions that usually terminate in death. During these convulsions, chicks may run aimlessly about, flapping their wings and falling to their sides or rolling completely over on their backs, where they perform rapid jerking motions with their feet and heads. These signs may be distinguished from those of encephalomalacia (vitamin E deficiency) by the relatively greater intensity of activity of the chicks during a seizure, which results in complete exhaustion and often death.

Gries and Scott (77) observed that chicks fed very low levels of pyridoxine (up to 2.2 mg B_6/kg diet) combined with a high protein level (31%) have classic nervous signs. Intermediate levels (2.5–2.8 mg B_6/kg diet) combined with 31% protein cause severe chondrodystrophy but no nervous signs. The consequence is bone curvature. If the diet contains 22% protein, even the lowest levels of pyridoxine (1.9 mg/kg diet) fail to induce nervous signs, chondrodystrophy, or even lowered growth rate. The function of pyridoxine in amino acid metabolism is reflected in an increased requirement when high levels of protein or methionine are fed (168). A pyridoxine deficiency causes a defect in collagen fibers in cortical bone and articular cartilage matrix and increased solubility of proteoglycans and collagen (128). These structural defects apparently cause chondrodystrophy and osteoarthritis in deficient chicks.

Clinical signs of pyridoxine deficiency in ducklings are reported to include poor growth and food consumption, hyperexcitability, weakness, microcytic hypochromic anemia, convulsions, and death (227).

In adult birds, pyridoxine deficiency causes marked reduction of egg production and hatchability, as well as decreased feed consumption, loss of weight, and death. The injection of pyridoxine into the fertile egg has increased the hatchability of eggs from turkey breeders that had received in their diets more than twice the concentration of pyridoxine estimated as the requirement by the National Research Council. This suggests that the requirement of breeders under some conditions may be higher than the dietary level of pyridoxine used under practical conditions.

Biotin

Biotin is a cofactor in carboxylation and decarboxylation reactions involving fixation of carbon dioxide. These reactions have important roles in anabolic processes and in nitrogen metabolism.

Clinical Signs, Signalment, and Pathology of Deficiency

In biotin deficiency, the dermatosis of the feet and skin around the beak and eyes is similar to that of pantothenic acid deficiency. Thus, in making a differential diagnosis, it is usually necessary to examine composition of the diet.

Chondrodystrophy is a sign of biotin avitaminosis in growing chickens and turkeys. Biotin deficiency signs in chicks include various other abnormalities of the tibia. Bain *et al.* (17) reported that chicks fed a purified diet devoid of biotin had shortened tibiae, higher bone density and bone ash, and an abnormal pattern of bone modeling: The median side of the mid-diaphyseal cortex was thicker than the lateral side in chicks fed the biotin-free diet, whereas the opposite pattern existed for chicks fed the same diet supplemented with adequate biotin. This raises the possibility that biotin may have a role in various deformities of the limb (17). Changes in tibial concentrations of fatty acids that are prostaglandin precursors correlate with bone abnormalities in biotin-deficient chicks, suggesting that altered prostaglandin synthesis may be a contributing factor in altered bone modeling patterns of the tibiotarsus in biotin deficiency (204).

Biotin is essential for embryonic development (47, 48). Embryos from hens fed biotin-deficient diets developed syndactylia, an extensive webbing between the third and fourth toes. Many embryos that fail to hatch are chondrodystrophic—characterized by reduced size, a parrot beak, severely crooked tibia, shortened or twisted tarsometatarsus, shortened bones of the wing and skull, and shortening and bending of the scapula. Two peaks of embryonic mortality may occur: one during the first week and a second during the last 3 days of incubation.

Robel and Christensen (160) reported that the injection of 87 μg of D-biotin into eggs of large white turkey hens that had been held under commercial conditions resulted in approximately 4–5% higher hatchability of their eggs. The reason for the improvement is not known; however, the authors suggest that biotin levels or biotin availability in the egg may have been low.

Fatty liver and kidney syndrome (FLKS) is a biotin-responsive condition that has been observed in broiler chicks. Chicks exhibit depressed growth; fatty infiltrations of liver, kidney, and heart; decreased plasma glucose; increased plasma-free fatty acids; and increased ratio of C16:1 to C18:0 fatty acids in liver and adipose tissue (150, 210). High dietary protein or fat reduces or eliminates mortality, whereas high protein or fat increases the signs of biotin deficiency. Fasting exacerbates FLKS and its associated mortality (210). Fasting decreases blood glucose concentrations and increases plasma-free fatty acids. Pyruvic carboxylase, a biotin-containing enzyme, is decreased in activity in FLKS biotin deficiency (150). It has been suggested that biotin deficiency impairs gluconeogenesis as a result of low activity of this enzyme, leading to increased conversion of pyruvate to fatty acids. Chicks having FLKS frequently do not have the characteristic signs of biotin deficiency. This may be a temporal phenomenon wherein the changes in tissue metabolism leading to FLKS occur rapidly in biotin-depleted chicks, but the classic signs of biotin deficiency require a longer period of time to develop (32).

Biotin has been suspected of having a role in "acute death syndrome" (or "sudden death syndrome") in broiler chickens. Biotin deficiency alters the unsaturated fatty acid profile in tissue lipids in such a manner as to suggest that it impairs the conversion of

linoleic acid to arachidonic acid (203). The latter is a precursor of the prostaglandins, prostocyclin I2 and thromboxane A2, which have marked effects on the vascular system. The concentration of biotin in liver was reported to be depressed in chicks that exhibited acute death syndrome (108). The role of biotin in acute death syndrome, however, remains obscure.

Biotin bioavailability for chickens and turkeys varies greatly among practical feed ingredients (69, 134, 211). Biotin is no more than 10% available in some grains but almost completely available in others. This is an important consideration in formulating diets to satisfy the biotin requirements of poultry.

Treatment of Deficiency

Patrick *et al.* (149) and Jukes and Bird (98) reported that injection or oral administration of a few micrograms of biotin was sufficient to prevent biotin deficiency signs in chicks and turkey poults.

Folic Acid (Folacin)

Folic acid is a part of the enzyme system involved in single-carbon metabolism. It is involved in synthesis of purines and the methyl groups of such important metabolites as choline, methionine, and thymine. Folic acid, therefore, is required for normal nucleic acid metabolism and formation of the nucleoproteins required for cell multiplication.

Clinical Signs, Signalment, and Pathology of Deficiency

Folic acid deficiency in chicks is characterized by poor growth, very poor feathering, anemia, and chondrodystrophy. Folic acid is required for pigmentation in feathers of Rhode Island red and black leghorn chicks. Thus, folic acid, lysine, copper, and iron appear to be required for prevention of achroma of feathers in colored poultry.

A deficiency in the breeding diet of chickens or turkeys causes a marked increase in embryonic mortality. Embryos die soon after pipping the air cell. According to Sunde *et al.* (186, 187, 188), a deformed upper mandible and bending of the tibiotarsus are lesions of embryonic deficiency. Poults show a characteristic cervical paralysis and die within 2 days after the onset of these signs unless folic acid is administered immediately. Poults show only a slight anemia.

Folic acid deficiency in chicks causes megaloblastic arrest of erythrocyte formation in bone marrow, which results in a severe macrocytic anemia as one of the first signs in chicks. White cell formation also is reduced, causing a marked agranulocytosis.

Folic Acid-Choline Interrelationship

Folic acid has a central role in methyl group metabolism. Young *et al.* (229) observed that when a diet for chicks is deficient in folic acid, an increase in the dietary level of choline reduces, but does not completely prevent, the incidence and severity of chondrodystrophy. A growth depression has been observed in chicks fed a practical diet that was low in folic acid and marginally deficient in methionine and choline. Supplementation of the diet with folic acid or methionine and choline stimulated growth under these conditions (152).

Treatment of Deficiency

A single intramuscular (IM) injection of 50–100 μg pure pteroylglutamic (folic) acid causes a peak reticulocyte response within 4 days in severely anemic folic acid-deficient chicks (161). Hemoglobin values and growth rates return to normal within 1 week. Addition of 500 μg folic acid/100 g feed caused recovery comparable to that obtained with injection of the vitamin.

Vitamin B_{12} (Cobalamin)

Vitamin B_{12} is involved in nucleic acid and methyl synthesis and carbohydrate and fat metabolism. One of its main enzyme functions involves isomerization of methylmalonyl coenzyme A to form succinyl CoA.

Clinical Signs, Signalment, and Pathology of Deficiency

Vitamin B_{12} deficiency results in slow growth, decreased efficiency of feed utilization, mortality, and reduced egg size and hatchability. Specific signs for vitamin B_{12} deficiency have not been demonstrated in growing or mature poultry. Vitamin B_{12} deficiency has been reported to cause myelin degeneration in chicks. Some investigators have detected increased total phospholipids and decreased levels of galactolipids from deficient chicks, suggesting impaired myelin maturation (102). Chondrodystrophy may occur in vitamin B_{12}-deficient chicks or poults when their diets lack choline, methionine, or betaine as sources of methyl groups. Addition of vitamin B_{12} may prevent chondrodystrophy under these conditions because of its effect on the synthesis of methyl groups.

Vitamin B_{12}-deficient embryos have a peak in mortality at day 17 of incubation, reduced size, myoatrophy of the legs, diffuse hemorrhages, chondrodystrophy, edema, and fatty liver (139, 145).

Treatment of Deficiency

Peeler *et al.* (151) showed that IM injection of 2 μg vitamin B_{12}/hen increased hatchability of eggs from vitamin B_{12}-deficient hens from approximately 15–80% within 1 week. Addition of 4 mg vitamin B_{12}/ton breeding ration is sufficient to maintain maximum hatchability and to produce chicks having sufficient stores of the vitamin to prevent any deficiency during the first few weeks of life. Similar injections of young chicks followed by supplementation of the chick ration also will correct the deficiency.

Choline

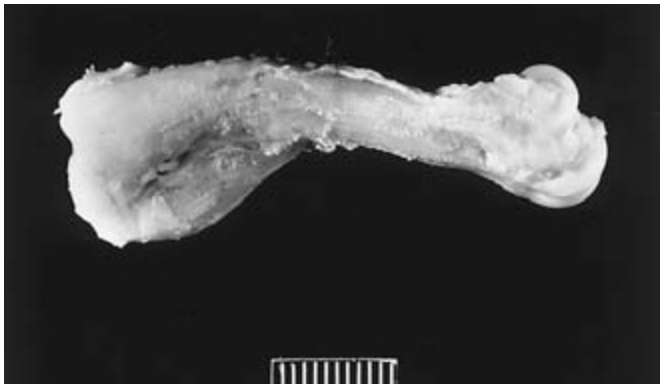
Choline is present in acetylcholine and body phospholipids. It acts as a methyl source in synthesis within the body of methyl-containing compounds such as methionine, creatine, carnitine, and N-methylnicotinamide. Choline per se does not act as a methyl donor but first must be oxidized to the compound betaine, which then can donate 1 of its 3 methyl groups to a methyl-acceptor such as homocysteine or glycocyamine for formation of methionine or creatine, respectively.

Clinical Signs, Signalment, and Pathology of Deficiency

In addition to poor growth, the most consistent lesion of choline deficiency in chicks and poults is chondrodystrophy (Figs. 29.10



29.10. Choline deficiency. Stunting; poor feathering; and short, thick, bowed legs typical of chondrodystrophy are seen in a bird that had been fed a choline-deficient diet. (Swayne)



29.11. Choline deficiency. Chondrodystrophy and deformity of tibiotarsus from broiler chicken given a diet lacking adequate choline. (Swayne)

and 29.11). Young turkeys have a high requirement for choline and, therefore, will show a high incidence of severe chondrodystrophy unless special care is taken to supplement the diet with choline. Chondrodystrophy is first characterized by pinpoint hemorrhages and a slight puffiness about the hock joint, followed by an apparent flattening of the tibiometatarsal joint caused by rotation of the metatarsus. The metatarsus continues to twist and may become bent or bowed until it is out of alignment with the tibia. When this condition exists, the leg cannot adequately support the weight of the bird. The articular cartilage is deformed and the Achilles tendon (tendo calcaneous) slips from its condyles.

When laying pullets that have received high-choline rearing diets are fed severely deficient diets, the percentage of fat in the liver increases. In livers of choline-deficient chickens, fat content is higher in females than males. Choline deficiency is, however, rare in adult chickens and turkeys fed practical rations. Nesheim *et al.* (138) showed that pullets fed high choline levels during the 8–20-week growing period are more likely to show fatty livers when placed on purified low-choline laying diets than pullets fed minimum levels during the same growth period. These results indicate that maturing chickens can synthesize choline but will not fully develop this ability if given diets containing ample amounts.

Treatment of Deficiency

If choline deficiency is noted in chicks or poults before severe signs of chondrodystrophy have developed, the deficiency can be cured by supplementing the ration with sufficient choline to meet the requirements. After the tendon has slipped in chicks or poults suffering from choline deficiency, the damage is irreparable.

Essential Inorganic Elements

Essential mineral elements are as important as amino acids and vitamins in maintenance of life, well-being, and production in poultry. Bone and eggshell owe their rigidity to calcium salts. Electrolytes regulate osmotic pressure and acid-base balance and exert specific effects on the ability of muscles and nerves to respond to stimuli. Minerals also are necessary as catalysts or for activation of many enzymes of the body and some are necessary components of macromolecules.

The minerals essential for maintenance of well-being are calcium; phosphorus; sulfur; magnesium; potassium; sodium; chlorine; and the trace elements manganese, iron, copper, zinc, iodine, molybdenum, chromium and selenium (142). Arsenic, boron, fluoride, nickel, rubidium, vanadium, and some rare earth minerals may also have essential functions, but mechanistic information is lacking. Analyses of individual mineral constituents in the body of chickens show that major portions of calcium, phosphorus, magnesium, and zinc are present in bones. Other essential elements are distributed largely in muscles, other soft tissues, and body fluids.

Calcium and Phosphorus

Calcium (Ca) and phosphorus (P) are closely associated in metabolism, particularly in bone formation. The major portion of dietary calcium is used for bone formation in growing chicks or poults and for eggshell formation in mature hens. Calcium also is essential for clotting of blood, and it is required along with sodium and potassium for normal contraction of cardiac muscle. Calcium is an integral constituent of cell signaling and regulatory pathways.

In addition to its role in bone formation, phosphorus is an essential component of purine nucleotides and other phosphorylated compounds involved in the transfer or conservation of free energy in biochemical reactions. It is an integral component of

many macromolecules and is involved in the regulation of many cellular and metabolic processes. Phosphorous also plays a critical role in the maintenance of acid-base balance.

Calcium and Phosphorus Deficiency

The utilization of calcium and phosphorus depends on presence of an adequate amount of vitamin D in the diet. In vitamin D deficiency, the deposition of these minerals in bones of growing chicks and poults is reduced; bones become depleted of mineral; and the quantity of calcium in eggshells is decreased.

According to Long and associates (121, 122, 123), deficiencies of calcium and phosphorus in the diet of growing broiler chicks cause rickets that differs in histopathology from the rickets of vitamin D deficiency. Tibiae from chicks that had been fed a diet containing 0.3% Ca from the time of hatching showed, by 2 weeks, a widening of the proliferating prehypertrophic zone of epiphyseal cartilage and irregular contours in the boundary between the zones of proliferating and hypertrophic cartilage (123). Irregular cartilage columns and elongated epiphyseal vessels were present. By 4 weeks, the epiphyseal growth plate had widened and, in some cases, extended as a cartilaginous plug into the metaphysis. Histologically, the proliferating and hypertrophic zones were irregular and often contained areas of nonviable cells. The hypertrophied zone was markedly widened in some chicks by 4 weeks. Metaphyseal blood vessels invaded along the lateral, but not the apical, region of the cartilaginous plug; cartilage columns of the metaphysis were thickened and irregular. The investigators note that the pathology is similar to that of tibial dyschondroplasia.

According to Long *et al.* (121), phosphorus deficiency (0.2% available dietary P) and calcium excess (2.24% Ca and 0.45% available P) resulted in similar abnormalities of the tibia. Several histologic abnormalities were observed, but most conspicuous was a marked lengthening of the cartilage columns of the degenerating hypertrophied epiphyseal cartilage and metaphyseal primary spongiosa. Some chicks were unable to stand at 4 weeks, displaying a spraddle-legged posture. Folding fractures and bowing or rotation of the tibiotarsus were frequently observed.

Julian (100) observed that phosphorus-deficient chicks had increased respiratory rates and were polycythemic. Blood CO_2 and O_2 were decreased, presumably due to poor rib strength and infolding, which interfered with respiratory movements of the rib cage. Birds died of right ventricular failure, often accompanied by ascites.

In laying hens, calcium deficiency results in reduced egg production and thin-shelled eggs as well as a tendency to deplete calcium content of the bones, first by complete removal of the medullary bone, followed by a gradual removal of the cortical bone. Finally, bones become so thin that spontaneous fractures may occur, especially in vertebrae, tibia, and femur. This condition may be associated with a syndrome commonly termed "cage layer fatigue" (156). Although a marginal calcium deficiency has often been found to be a triggering agent in cage layer fatigue, the syndrome apparently is not due to a simple deficiency of calcium but also involves other etiologic factors not yet identified.

The phosphorus in plant-base feedstuffs is poorly available because much of it is present in phytic acid and is not released by digestive enzymes. The availability of phosphorus can be increased by the inclusion in the diet of phytase of microbial or plant origin.

Excess Calcium

Shane *et al.* (177) fed leghorn pullets diets containing 3.0% Ca and 0.4% P from 8–20 weeks of age. Nephrosis and visceral urate deposition (e.g., "visceral gout") were observed in the high calcium treatment by 16 weeks of age. Wideman *et al.* (213) provided replacement pullets diets containing excess (3.25%) or adequate (1.0%) levels of calcium in combinations with moderate (0.6%) or low (0.4%) available phosphorus from 7–18 weeks of age. All birds received a commercial layer diet during the laying period. Pullets fed on the 3.25% Ca diets developed a high incidence of urolithiasis by 18 weeks, which persisted or increased in the laying period through 51 weeks of age. Low levels of dietary phosphorus during the rearing period exacerbated the effect of excess calcium.

Magnesium

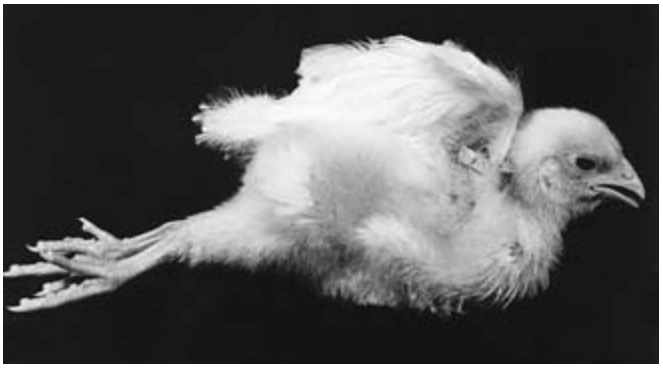
Magnesium (Mg) is essential for carbohydrate metabolism and for activation of many enzymes, especially those involved in phosphorylation reactions. It is essential for bone formation, about two-thirds being present in bone chiefly as a carbonate. Eggshells contain about 0.4% Mg.

Deficiency

Almquist (7) observed that chicks fed a magnesium-deficient diet grew slowly for approximately 1 week and then ceased growing and became lethargic. When disturbed, these chicks frequently passed into a brief convulsion accompanied by gasping and finally into a comatose state sometimes ending in death. Magnesium deficiency signs of poults are similar to those of chicks (185). Hypomagnesemia and hypocalcemia are associated with severe magnesium deficiency in chicks. Tibiae have decreased magnesium and increased calcium content and exhibit abnormalities (185, 205, 206) including thickening of trabeculae, increased retention of cartilage cores, and the occurrence of elongated and inactive osteocytes in the metaphysis. Deficient chicks have thickening of the cortex, the presence of elongated inactive osteocytes, and enlargement of Haversion canals within the diaphysis. The epiphyseal plate, however, appears normal. The parathyroid appears hyperactive, perhaps in response to the hypocalcemia that is characteristic of magnesium deficiency (206).

Excess Magnesium

Ordinary feeds supply enough magnesium in practical poultry diets to meet requirements. It is possible, however, that under certain conditions rations may contain excess magnesium, producing detrimental effects including reduced growth rate and bone ash in chicks and decreased egg size, eggshell thinning, and diarrhea in hens (43, 132, 184). The maximum tolerable level of magnesium in the diets of poultry is about 0.5% for growing birds and 0.75% for laying hens (142). Diets with low levels of phosphorus enhance the sensitivity of hens to toxicosis (85).



29.12. Characteristic sign of chloride deficiency. (Leach)

Sodium and Chlorine (Salt)

Sodium (Na) as chloride (Cl), carbonate, and phosphate is found chiefly in blood and body fluids. Sodium is connected intimately with maintenance of membrane potentials, cellular transport processes, and the regulation of the hydrogen ion concentration of blood. Chloride, the major mineral anion in extracellular fluid, plays a role in fluid, ionic balance, and acid-base balance.

Clinical Signs and Signalment of Deficiency

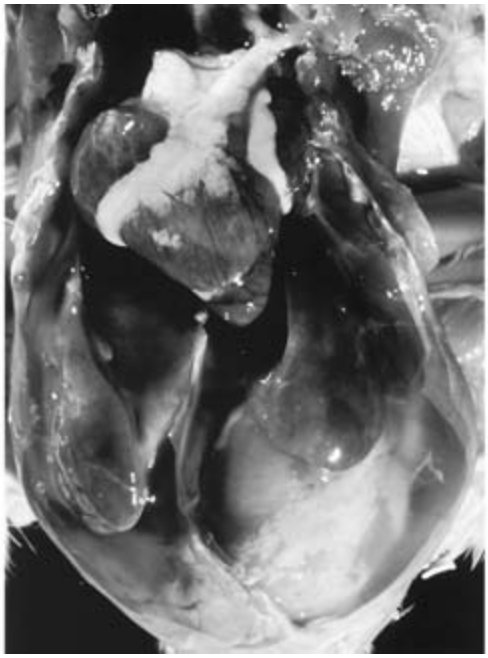
Animals receiving diets deficient in sodium not only fail to grow but also develop softening of bones, corneal keratinization, gonadal inactivity, adrenal hypertrophy, changes in cellular function, impairment of food utilization, and decrease in both plasma and special fluid volumes. Cardiac output drops; mean arterial pressure falls; the hematocrit increases; elasticity of subcutaneous tissue decreases; adrenal function is impaired; and a state of shock results, which if uncorrected, terminates in death.

Chicks fed a diet containing no added salt show retarded growth with decreased efficiency of food utilization. Lack of salt in the diet of laying hens results in an abrupt decrease of egg production and reduced egg size, loss of weight, and cannibalism.

Salt deprivation in turkeys impairs egg production and hatchability (81). Leach and Nesheim (115) observed that chicks fed a purified diet containing 0.24% Na and 0.4% potassium required 0.12% chlorine. They produced chloride deficiency by feeding young chicks a purified diet containing 190 mg Cl/kg diet. Chicks exhibited extremely poor growth rate, high mortality, hemoconcentration, dehydration, and reduced blood chloride. In addition, deficient chicks showed nervous signs characteristic of Cl deficiency. When startled, they fell forward with their legs outstretched behind them and lay paralyzed for several minutes, then appeared quite normal until frightened again (Fig. 29.12).

Excess Salt

Large amounts of salt in the ration are toxic to chickens and turkeys. The lethal dose is approximately 4 g/kg body weight. Young chicks appear to be more susceptible to toxic effects of salt than are older chickens. Poultry are much less tolerant to salt supplied via the water than by the diet. Signs of salt intoxication include inability to stand, intense thirst, pronounced muscular weakness, and convulsive movements preceding death. There are lesions in many organs, particularly hemorrhages and severe congestion in the gastrointestinal tract, muscles, liver, and lungs. Excess sodium results in ascites, right ventricular hypertrophy, and right ventricular failure in broiler chickens (Fig. 29.13) (99, 202, 225) and contributes to spontaneous cardiomyophathy in turkeys (68). Matterson *et al.* (129) fed day-old poults graded quantities of salt for 23 days and observed 25% edema and 25% mortality at 4.0% salt but none at 2.0%. Swayne *et al.* (190), however, described a case of accidental salt poisoning in 5–11-day-old poults in which a diet contained 1.85% salt. Signs included respiratory distress, ascites, hydropericardium, hydrothorax, and sudden death. High levels of salt also cause the excretion of dilute urine and wet litter.



29.13. Sodium excess. Cardiomegaly, especially involving the right ventricle, ascites, and fibrin masses in the body cavity and on the liver capsule, occurred in this chicken given excess sodium. (Swayne)

Potassium

Potassium is found primarily in the cellular compartment of the body; soft tissues of the chicken contain more than 3 times as much potassium as sodium. As a major cation in intracellular fluid, potassium has an essential role in the maintenance of membrane potential and cellular fluid balance. Potassium participates directly in numerous biochemical reactions and is necessary for normal heart activity, reducing contractility of the heart muscle and favoring relaxation.

Clinical Signs and Signalment of Deficiency

The main effect of potassium deficiency is overall muscle weakness characterized by weak extremities, poor intestinal tone with distention, cardiac weakness, and weakness of the respiratory

muscles and their ultimate failure. Severely affected individuals may exhibit tetanic seizures followed by death. Low levels of potassium in laying diets cause decreased egg production and eggshell thinning (113). A low potassium level in the vital organs of animals may occur during severe stress. Plasma potassium is elevated, causing the kidney (acting under influence of the adrenocortical hormone) to discharge potassium into the urine. During adaptation to stress, the muscle will begin to retrieve its lost potassium. As liver glycogen is restored, potassium returns to the liver. This may result in temporary prolongation of the general potassium deficiency throughout the body. High temperature results in increased loss of potassium in the urine (54).

An all-vegetable diet low in potassium produced low plasma potassium concentrations and a high incidence of sudden death syndrome at the onset of egg production in broiler breeder pullets that had undergone restricted feeding (91). The hypothesis that low dietary potassium leads to this syndrome, however, has not been subjected to testing.

Dietary Balance of Macrominerals

Studies in many laboratories during the past 2 decades have determined that the balance among dietary minerals has a profound effect on acid-base balance and certain developmental, metabolic, and physiologic functions in poultry (135). Balance has been expressed in several ways. One expression is dietary undetermined anion (dUA), sometimes referred to as mineral cation-anion balance (13). It is defined as follows: dUA = (Na+K+Ca+Mg) – (Cl+P+S), in which all values are expressed in milliequivalents per kg of diet and valences are assumed to be +1 for Na and K, +2 for Ca and Mg, –1 for Cl, –1.75 for P and –2 for S. P and S are assumed to be inorganic. Trace minerals are excluded because of their insignificant contributions to the overall mineral balance. Another term, dietary electrolyte balance, emphasizes the balance among the strong electrolytes (Na+K-Cl).

A positive value of dUA represents the net dietary concentration of organic anions. If the value is negative, a very unusual condition, it is a measure of the net hydrogen ion content of the diet. Minerals differ in their chemical properties and metabolism. Therefore, although dUA provides an indication of the qualitative effect, it is not an accurate predictor of the quantitative effect of the diet on acid-base balance.

Diets rich in mineral anions, particularly Cl, tend to cause metabolic acidosis and result in disturbances of Ca metabolism, increased incidence and severity of tibial dyschondroplasia in immature fowls, and reduced eggshell calcification in laying hens. Effects on tibial development (60, 79, 116, 167) and eggshells (14) are exacerbated when calcium is limiting.

A dietary combination of excessive calcium and low phosphorus results in the excretion of an alkaline urine (213), as would be predicted from dUA. The urolithiasis observed in replacement pullets by Wideman *et al.* (213) under these conditions may be due in part to the increased pH of urine. Alkaline conditions favor the precipitation of divalent mineral salts. Growing chicks given high levels of sodium bicarbonate develop visceral urate deposition (e.g., "visceral gout") that is especially pronounced in the kidney, which displays urate granulomas (tophi) in renal interstitium and tubular necrosis (137). Increasing the dietary acid load has been used to reduce uroliths (213) in poultry and some mammals (29). The potentially adverse effects of low dUA on bone development and eggshell quality should be considered before such treatment is attempted.

High levels of dietary electrolytes also increase fecal moisture and can cause problems with wet litter. Increasing dietary concentrations of sodium, potassium or phosphorus causes linear increases in the water intake of the laying hens and linear increases in the moisture content of their excreta. Each 1 g/kg increase in dietary mineral increased the moisture content of the excreta by 9.0, 12.0 and 5.6 g/kg for sodium, potassium and phosphorus, respectively (179). Various sodium salts ameliorate heat stress in chickens, at least in part by increasing water intake (6, 29).

Manganese

Manganese (Mn) is an activator of several enzymes and is required for normal growth and reproduction and prevention of chondrodystrophy. In addition to its chondrodystrophy-preventing properties, manganese is necessary for formation of normal bones. Wilgus *et al.* (218) observed that leg bones of chicks fed chondrodystrophy-producing diets frequently were thickened and shortened. Manganese deficiency impairs endochondral bone growth. Cells of the epiphyseal growth plate are arranged irregularly and the extracellular matrix is greatly reduced (112). Manganese is essential for the activity of glycosyltransferases; a deficiency of manganese impairs the synthesis of the glycosaminoglycan molecules that are components of proteoglycans in the cartilage of the epiphyseal growth plate (114, 120). Bone of manganese-deficient ducks, consequently, contains a reduced concentration of hexosamine (114). Manganese also has been reported to be necessary for maximum eggshell quality.

Lyons and Insko (126) found that manganese deficiency resulted in very low hatchability of fertile eggs and chondrodystrophy in embryos. The peak of mortality for such embryos occurred on day 20 and 21 of incubation. Chondrodystrophic embryos were characterized by very short, thickened legs, short wings, parrot beak, globular contour of head, protruding abdomen, and retarded down and body growth. Marked edema was noted in about 75% of these embryos. The manganese content of eggs producing chondrodystrophic embryos was less than that of normal eggs.

Chicks hatched from eggs produced by hens fed a diet deficient in manganese sometimes exhibit ataxia, particularly when excited (38). The head may be drawn forward and bent underneath the body or retracted over the back. Ataxic chicks grow normally and reach maturity but fail to recover completely. They also retain the short bones characteristic of embryos and newly hatched chicks from manganese-deficient dams (37).

Manganese is generally considered to be one of the least toxic minerals and poultry tolerate diets with up to 2,000 mg/kg without signs of toxicosis (142).

Iodine

Traces of iodine (I) are required for normal functioning of the thyroid gland in poultry as in other animals. Thyroxine contains

approximately 65% I and acts as an important regulating agent in body metabolism. When the intake of iodine is suboptimal, the thyroid tissue enlarges and goiter results.

Wilgus *et al.* (217) reported that iodine deficiency results in an enlarged thyroid and, in some cases, lower body weight in growing chicks. They observed congenital goiter in hatchlings from hens receiving 0.025 ppm I in the ration. Rogler *et al.* (162) observed mortality late in incubation. Hatching time was delayed. Embryo size was reduced and yolk sac resorption was retarded. Use of 0.25% iodized salt in chicken and turkey rations should prevent development of iodine deficiency. This would supply 0.175 ppm in addition to that contained in the diet. Christensen and Ort (45) reported that dietary supplements of iodine increased the permeability of eggshells and the hatchability of turkey eggs.

Iodine deficiency in poultry has been largely prevented by widespread use of iodine either in iodized salt or as part of the trace mineral premix. Some egg producers feed high levels of iodine in order to enrich eggs and provide added value. Levels of dietary iodine of 12 mg/kg and above decrease egg weight, albumen index and Haugh units (226). Lichovnikova *et al.* (119) observed decreased egg production, size, and Haugh units at 6.01 mg of iodine/kg diet. Iodine reduces the growth rate of chicks at 900 mg/kg and this effect is mitigated by dietary bromine (19). High levels of iodine also reduce male fertility. Iodine toxicosis is normalized within about 7 days of returning birds to a diet with normal iodine levels (118).

Copper

Copper (Cu) is essential for formation of hemoglobin. In the absence of copper, dietary iron is absorbed and deposited in the liver and elsewhere but hemoglobin synthesis does not occur. Copper deficiency in chicks results in anemia, characterized by reduced numbers of circulating erythrocytes, and impaired feather pigmentation in colored breeds of fowl (75).

Copper is a component of several enzymes that participate in redox reactions. Lysyl oxidase is a copper-containing enzyme that catalyzes oxidation of lysine residues in formation of the cross-linking structure desmosine in elastin (164). Copper deficiency decreases the cross-linking. This weakens the structure of elastin, leading to aortic rupture in poultry. Thinning of the tertiary bronchial mantle in lungs may also result from decreased cross-linking of elastin (33); however, observations on birds fed high levels of cadmium appear inconsistent with this view (117). Copper deficiency has been reported to decrease cross-linking in bone collagen and to increase bone fragility (146, 163). Copper is a component of superoxide dismutase, cytochrome oxidase, and ceruloplasmin—all of which have decreased activities in copper-deficient chicks (28, 107). A copper deficiency also causes hypertriglyceridemia, hypercholesterolemia, and prolonged prothrombin time (104). A deficiency of copper in laying hens causes reduced egg production, increased egg size, and abnormal eggshell calcification. Eggshell abnormalities include shell-less eggs, misshapen eggs, wrinkled eggshells, and reduced eggshell thickness. The palisade layer of the eggshell appears normal; however, the mammillary layer has enlarged mammillary knobs and increased spacing between knobs. This may be related to an abnormal structure of eggshell membranes caused by a decrease in lysine cross-linking (23).

Relatively high levels of copper (100 to 200 mg/kg diet) are often fed to poultry because of an antibiotic-like effect that improves growth and efficiency. Growth promoting levels of copper decrease the number of lymphocytes in the lamina propria of the intestines and also decrease the numbers of intraepithelial lymphocytes (9).

Excess Copper

Excessive dietary levels of copper have been reported to cause abnormalities of the ventriculus. Fisher *et al.* (67) reported that dietary copper levels ranging from 205–605 ppm resulted in a rough thickened ventricular lining in broiler chicks, the severity of the lesion increasing as the copper level of the diet increased. The highest copper level caused markedly thickened and folded linings having a warty appearance. Histologic examination revealed thickening of the koilin layer, sloughing of epithelial cells into the area under the koilin layer and the inclusion of clusters of sloughed cells within the koilin layer. Wight *et al.* (216) observed similar lesions in chicks receiving 2000 and 4000 ppm copper. They noted ventricular erosions and fissures in the ventriculus lining, hemorrhages under the koilin layer, and a mucoid material adhering to the mucosa of the proventriculus. Chiou observed liver pathology, including proliferation of bile ducts and infiltration of lymphocytes, and elevated serum creatine kinase, aspartate amino transferase, and lactate dehydrogenase in laying hens fed 800 ppm copper (44). Ducks are more sensitive to copper toxicosis than chickens (142).

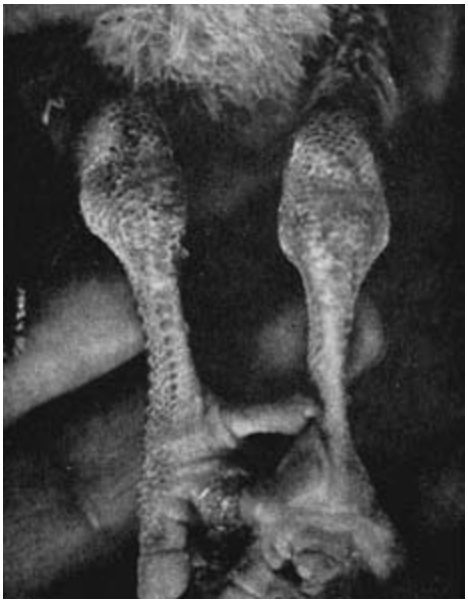
Iron

Iron (Fe) is an essential component of heme, the porphyrin nucleus of hemoglobin and the cytochromes, and is a component of several enzymes including catalase, peroxidase, phenylalanine hydroxylase, tyrosinase, and proline hydroxylase.

Iron deficiency results in a hypochromic, microcytic anemia and reduced concentration of nonheme iron in plasma and prevents normal feather pigmentation in breeds having colored plumage (52, 86). A deficiency in laying hens also causes anemia in the developing chick embryo and reduced hatchability (136). Chicks that survive incubation are weak and listless; however, they recover when given supplemental iron.

The hemoglobin level of hens falls with the beginning of egg production, but this apparently is not related to the iron or copper content of the diet. Since the hemoglobin level rises rapidly with onset of broodiness, it is more probable that low levels prevailing in egg production are caused by changes in hormone activity rather than iron or copper deficiencies. A deficiency of iron has been reported to decrease the synthesis of niacin from tryptophan in chicks (144).

Characteristic signs of chronic iron toxicosis include decreased growth and efficiency of gain. Cao *et al.* (36) observed slightly decreased growth in day old chicks fed practical diets supplemented with 400 mg iron/kg diet as $FeSO_4$ and 800 mg/kg resulted in poor growth. However, if iron supplemented diets



29.14. Enlarged hocks in poult caused by zinc deficiency. (Young)

were introduced after day 5, performance of chicks fed 800 mg/kg supplemental iron was normal. The liver, heart, and pancreatic beta cells are most affected by iron overload (142).

Zinc

Traces of zinc (Zn) appear to be necessary for life in all animals. It is a constituent of the enzyme carbonic anhydrase and is an activator or a cofactor of more than 299 enzymes. Zinc stabilizes "zinc finger" structural motifs, which are involved in protein-DNA interactions that regulate gene expression.

Clinical Signs and Signalment of Deficiency

Deficiency of zinc results in retarded growth; poor feathering; enlarged hocks (Fig. 29.14); short, thickened long bones (chondrodystrophy); scaling of the skin and dermatosis, particularly on the feet; and an awkward arthritic gait (143, 228). Zinc-deficient chicks exhibit increased hematocrit, which is due to redistribution of body water rather than altered water intake (26, 27).

Histologic lesions include hyperkeratinization of skin of the shank and feet and parakeratosis of the esophagus. Nucleoli of the crop epithelium are enlarged and contain increased amounts of RNA. Alkaline phosphatase and alcohol dehydrogenase, two zinc-containing enzymes, exhibit reduced activities in the crop and esophagus (215). Severe epiphyseal growth plate lesions are characterized by reduced cellularity and abnormally shaped cells in areas remote from blood vessels. Changes in growth plate cellularity are due to decreased chondrocyte proliferation and increased cell apoptosis (108). Reduced alkaline phosphatase activity is also observed in epiphyseal cartilage. Starcher *et al.* (182) found that activity of the zinc-dependent enzyme collagenase is reduced in the tibia during zinc deficiency. They suggest that effects of zinc on bone may be the result of decreased bone collagen turnover. Lymphoid organs become depleted of lymphocytes and reticular cells of the thymus become necrotic (51). The effect of deficiency is greatest on the bursa, followed by thymus, and then spleen. Bettger *et al.* (26, 27) reported evidence of an interrelationship between vitamin E and zinc. Leg abnormalities, arthritic gait, and epidermal lesions were reduced by vitamin E and exacerbated by polyunsaturated fatty acids.

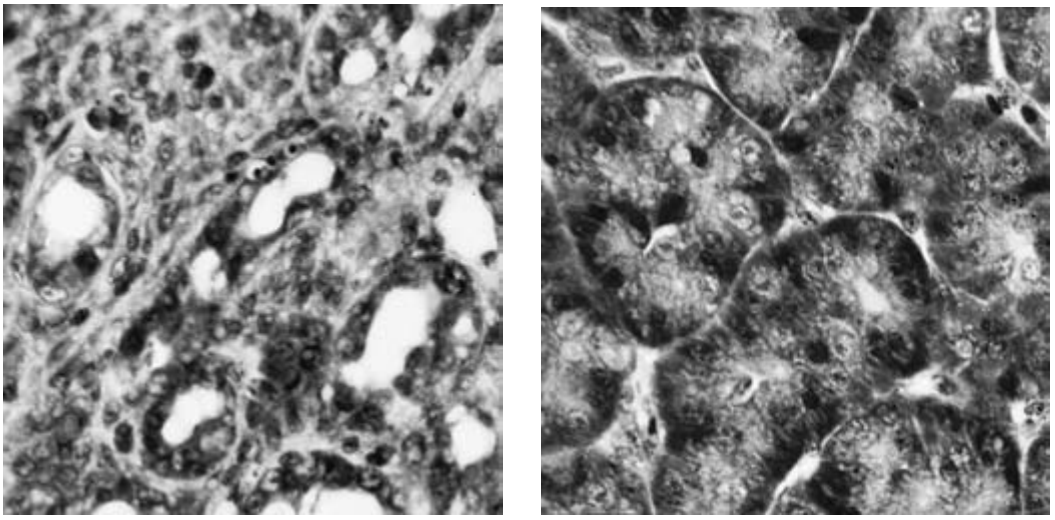
Ducks exhibit poor growth and epidermal lesions of the feet, particularly interdigital webs (214). Pathology of the epidermis is evident in the interdigital web, mucous membrane of the tongue, and epithelium in other parts of the gastrointestinal tract. Hyperkeratosis and acanthosis characterize the tongue and interdigital web lesions. Intercellular spacing between prickle cells and basal cells is increased and number of desmosomes is diminished. Prickle cells have an abnormal structure, enlarged nuclei and nucleoli, and decreased content of free ribosomes, tonofilaments, and other structures. Lymphoid organs are affected similarly to those of chickens (50).

The zinc requirement of poults is higher than for chicks. Thus, poults are more likely to show enlarged hocks and poor feathering of zinc deficiency unless special supplements are added to the diet. The most dramatic embryonic abnormalities resulting from nutritional deficiency appear when the breeding diet contains excess calcium and phosphorus, is high in phytic acid, and is deficient in zinc. Zinc-deficient embryos may have only a head and complete viscera but no spinal column beyond a few vertebrae, and no wings, body wall, or legs (106).

Excess Zinc

Excessive dietary levels of zinc (e.g., 20,000 ppm as zinc oxide) induce molt in laying hens (49). Zinc results in abrupt decline in egg production and onset of molt followed by rapid resumption of egg laying after dietary zinc concentrations are returned to normal. Excess zinc results in inanition, which is presumably responsible for initiating the molt (130). High levels of zinc result in accumulation of zinc in tissues and pathologic changes in the ventriculus, thyroid and pancreas. Chicks exhibit a rough, pale ventricular lining, which may show evidence of fissures and, less frequently, ulceration (56, 216). Histologic examination reveals epithelial desquamation and infiltration of inflammatory cells. Pancreases exhibit dilated acinar lumina and degenerative changes in acinar cells. The latter include loss of zymogen granules, vacuolization of the cytoplasm, the presence of hyaline bodies and other electron-dense debris (216). Follicular cell hypertrophy and hyperplasia occur in the thyroids (105).

Large excesses of dietary zinc such as those used to induce a molt result in reduced activity of the selenium-dependent enzyme, plasma glutathione peroxidase. Selenium administration restores glutathione peroxidase activity but fails to prevent pathologic changes in the ventriculus and pancreas (216). Lesser excesses of zinc (i.e., up to 2000 ppm) did not affect plasma or hepatic glutathione peroxidase activity, but interfered with exocrine function of the pancreas and reduced the plasma and tissue concentrations of a-tocopherol in chicks fed a purified diet but not in chicks fed a practical diet (124, 125).



29.15. Pancreas from selenium-deficient chick. Acini consist of degenerating cells forming central lumen with extensive interstitial fibrosis (left). Control (right). ×250. (Scott)

Selenium

Selenium has been shown to be an essential mineral element for both chicks and poults. It is a constituent of the enzymes, glutathione peroxidase and phospholipid hydroperoxide glutathione peroxidase, which serve to protect tissues against oxidative damage, and it is a component of iodothyronine 5′-deiodinase, an enzyme that is involved in the conversion of thyroxine to its active form (34).

Clinical Signs and Signalment of Deficiency

Selenium prevents development of exudative diathesis in young chickens and myopathy of ventriculus and heart in young turkeys (170, 171, 173). Selenium-deficient ducklings have reduced plasma glutathione peroxidase activity and exhibit low body weight gain and increased mortality. Ducklings that succumb to selenium deficiency may exhibit necrosis of several tissues including the ventriculus, heart, skeletal muscle and smooth muscle of the intestine, and show signs of hydropericardium and ascites (53). Vitamin E and selenium have a mutual sparing effect in prevention of these diseases (see Vitamin E). Pheasants display degenerative cardiomyopathy, vacuolar degeneration of hepatocytes, and centrilobular hepatic necrosis (110).

Chicks severely deficient in selenium exhibit poor growth and feathering, impaired fat digestion, pancreatic atrophy, and fibrosis (193, 194), and reduced activity of selenium-dependent glutathione peroxidase activity in the pancreas (207). Selenium deficiency depresses growth of broilers by inhibiting hepatic 5′-deiodinase activity, which causes lower plasma 3,5,3′-triiodothyronine concentration (94). Replenishing triiodothyronine normalizes growth and feed efficiency. Gries and Scott (76) performed a time-sequence study of pancreatic lesions, which began at 6 days of age with vacuolation and hyaline body formation in the exocrine pancreas. As the deficiency progressed, cytoplasm degenerated until acini were represented by rings of cells with a central lumen embedded in fibrous tissue (Fig. 29.15).

Treatment

Addition of 0.1 ppm Se as Na_2SeO_3 to the diet caused complete pancreatic acinar regeneration within 2 weeks and a marked clinical recovery. Injecting chicks displaying exudative diathesis with 15 μg selenium greatly improved clinical signs within 6 days (22). High dietary levels of vitamin E (15–20 times the amount needed for prevention of other vitamin E-deficiency diseases) protect against the pancreatic degeneration caused by selenium deficiency (207). High plasma tocopherol levels were maintained by feeding 100 IU vitamin E/kg and bile salts to enhance its absorption. This greatly reduced incidence of exudative diathesis, which did not appear until the pancreas in chicks had degenerated severely.

Excess

Excess inorganic selenium interferes with sulfur metabolism due to the formation of sulfur-selenium complexes and the substitution of selenium for sulfur in cysteine. Excess organic selenium, usually as selenomethionine, is incorporated readily into proteins because $tRNA_{met}$ does not distinguish selenomethionine from methionine and selenomethionine is readily incorporated into proteins in place of methionine. These aberrations result in impaired protein synthesis, impaired function of proteins, and mutagenesis. A decrease in growth rate occurs with 5 mg/kg selenium in broiler chicks (196). Salyi *et al.* (166) reported an incidence of acute selenium toxicity in broiler chicks that was evident by watery diarrhea, weakness, somnolence, and cerebellar edema. Hepatic lesions include vacuolic degeneration, pyknosis of cells of the mononuclear phagocytic system (MPS), hemorrhagic dystrophy, and parenchymal atrophy. Kidneys had diffuse tubulonephrosis, followed by the necrosis of tubular epithelium. Myocardial and skeletal myodegeneration and damage of the bursa of Fabricius were also observed.

The developing embryo is particularly affected by high selenium (219). Hatchability is typically poor and those that hatch are often deformed, often with legs, toes, wings, beaks, or eyes that are rudimentary or completely lacking. Down is often wiry

and sparse (84). Chicks consuming excessive levels of selenium display slow growth, pectoral muscle atrophy, hepatotoxicity, edema, and claw and feather loss.

Public Health Significance

Poultry products are an important part of the human diet and supply highly bioavailable forms of nutrients. Levels of vitamins and minerals in meat and eggs are highly dependent on the levels in the diet. Birds fed diets that are deficient in vitamins or minerals do not supply intended levels of nutrition to human consumers. Animals deficient in nutrients are often immunocompromised, resulting in increased incidence of infectious diseases and, in some cases, evolution of more pathogenic disease organisms.

Often animals can serve as buffers for high levels of minerals or other nutrients found in plants and other foodstuffs, thereby reducing human exposure to potentially toxic nutrients. However, levels of some nutrients (e.g. selenium, iodine, copper, fluoride, and vitamin A) may accumulate in meat or eggs to levels that might adversely affect human health (142). Prompt diagnosis and correction of toxicities is important for safeguarding the human food supply.

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Chapter 30

Developmental, Metabolic, and Other Noninfectious Disorders

Rocio Crespo and H. L. Shivaprasad

Introduction

The diseases and conditions discussed in this chapter represent a heterogeneous group; in some cases the etiology is quite clear, whereas in others it is questionable or unknown. They vary in economic importance and frequency of occurrence. Emphasis has been placed on metabolic diseases of economic importance to the modern poultry industry. Diseases have been classified by body system primarily affected; however, the initial part of this chapter covers diseases that are not associated with a specific body system conditions such as cannibalism, environmental disease, or amyloidosis.

Feather Pecking and Cannibalism

Feather pecking is a behavior expressed by dominant birds at subordinates. Feather pecking may vary from pecking to plucking the feathers of subordinate birds. Birds with damaged feathers have poor thermoregulation and greater energy demands than unaffected birds (356). The egg production in affected laying hens usually drops. If the feathers are severely damaged, hemorrhage may occur, which then attracts even more pecking. Appearance of blood on the exposed skin may lead to the death of the bird due to cannibalistic behavior from the other birds in the flock or the bird has to be culled due to the severity of the injuries.

There is a recognized individual and strain difference in the incidence of feather pecking, which implies a genetic component. Traditionally, light breeds of the Mediterranean class have been much more prone to feather pecking than the heavier breeds of the American and Asiatic classes (471). Today, cannibalism is more common in the modern brown hybrids than in the white layer lines (409). Recent studies have demonstrated genetic links between feather pecking and feather pigmentation (237).

Vent pecking appears to be a separate behavior. It generally occurs soon after birds have come into lay and may be linked to hormonal changes (294, 409). It is more common when birds in floor systems lay their eggs on the floor in crowded areas. It occurs immediately after oviposition, and exposure of the mucous membrane stimulates pecking by other birds. Vent pecking is responsible for at least 80% of all prolapses (153). It has been also hypothesized that vent pecking may be the initiating lesion that triggers the onset of salpingitis in the oviduct and perhaps egg peritonitis (349).

Etiology

There is not general agreement in the causes of feather pecking. Feather pecking may reflect redirected ground pecking; however, it is not clear whether it is related to foraging behavior (food searching and food consumption) or dust bathing (409). Other possible causes of feather pecking may be fearfulness (46, 196) and may also be related to accelerated sexual maturity and increased egg production (73). Feather pecking has been also associated with early sexual maturation, fast growth, and weak bones (211). This study also found that victims grew faster, had lower corticosterone levels, and were less active in a restraint test. Feather pecking is more common in females than males (211), and the presence of males is an important factor in reducing the problem (153). Some of the conditions reported to stimulate feather pecking are bright light, pelleted feed or compressed feed, high-density rearing systems, nutritional and mineral deficiencies, and irritation from external parasites (200, 339). Although feather pecking and feather damage are more severe in hens housed in cages than in floor systems, cannibalism is less likely to occur in hens housed in cages than it is in hens housed in pens (29, 411). Since feather pecking and cannibalism tend to reoccur within the same group or in adjacent cages, it is suggested that it may be a learned behavior (450).

Prevention

Outbreaks of feather pecking and cannibalism occur unpredictably in birds, despite the measures taken to reduce their risk. Feather pecking and cannibalism can best be prevented by providing adequate diet, supplying with mash diets rather than pelleted feed, rearing the birds on floor litter rather than slats, reducing light intensity, providing perches as a refuge for pecked birds, and avoiding overcrowding (15, 215, 409). Environmental enrichment with pecking devices can reduce many of the harmful effects of pecking and allows the birds to exercise their natural behavior (216). Avoid selecting pecking devices that can increase social pecking such as plastic rods or shoe laces; also do not use systems that may be ignored by birds such as beads or motorized devices. Simple devices such as hanging white or yellow strings are particularly attractive stimuli to chickens (217). Inclusion of oat hulls and other insoluble dietary fiber in diets decreases the

Grateful acknowledgment is made to Dr. C. Riddell, previous author of this chapter, for much of the material on reviews of several conditions, and many of the figures.

incidence and severity of feather pecking (184). Beak trimming has been recommended as a means for controlling feather pecking and cannibalism (200); however, it may not be totally effective. In addition, there is an increasing public concern that break trimming may cause chronic pain (74). Beak trimming also may stimulate the growth of neuromas in the stump of the beak if done after 5 weeks of age (197). Plastic devices have been used in preventing feather pecking in gamebird rearing (15, 135), but so far these devices are not satisfactory for use in domestic fowl (410).

Environmental Disease

Heat Stress

All classes of poultry experience heat distress when high temperatures accompanied by high humidity rise above their comfort zone. Birds, unlike mammals, do not have sweat glands. When the environmental temperatures are between 28°C and 35°C (82°F and 95°F), birds utilize non-evaporative cooling (radiation, conduction, and convection) as major means of heat dissipation. Birds manipulate non-evaporative cooling in two ways: 1) increasing the surface area by relaxing the wings and hanging them loosely at their sides, and 2) increasing the peripheral blood flow (492). As the environmental temperature approaches the body temperature of the bird (41°C or 106°F), the rate of respiration increases and the bird open-mouth breathes in order to increase evaporative cooling or water evaporation. If panting (open-mouth breathing) fails to prevent body temperature from rising, birds become listless, then comatose, and soon die due to respiratory, circulatory, or electrolyte imbalances (448).

Increased respiration rate alters the acid-base balance since the CO_2 concentration in the blood decreases (34). The higher blood pH reduces the amount of ionized calcium in the blood, which is needed for eggshell formation. In consequence, laying flocks experience an increase in the number of thin-shell eggs. Panting or open-mouth breathing in heat stressed birds may lead to an increased incidence of respiratory infections, since the natural filters of the nasal passages are bypassed. Another sign of heat stress in a flock is reduced feed intake. In growing birds, fasting will decrease growth rate. In laying flocks reduction of feed intake will result in reduction of egg size, egg production and egg quality.

The extent of the losses from heat stress are determined by the age, environmental history, the maximum temperature to which the bird is exposed, the duration of the high temperatures, the rate of temperature change, and the relative humidity of the air (163).

Every effort should be made to prevent or alleviate heat stress in the flock. Increased air circulation in the house can be done by running ventilation equipment at full capacity. Cooling the air inside the house can be accomplished by using sprinklers or spraying down the floor, walls, ceiling, and outside roof with cool water during times of extremely high temperature. During periods of high temperature, adequate drinking water should be available. Techniques aimed at lowering water temperature of heat-distressed birds will aid in heat dissipation.

Several diet manipulations have been tested in heat stress birds. Egg production improves by increasing the intake of protein relative to energy, but energy requirements increase in severe heat stress (12). Addition of vitamins (A, C, and E) and electrolyte supplements may help in preventing heat stress (439), possibly by replenishing the minerals and correcting the acid/base balance. However, there is still controversy regarding the effect of some these nutrients. For instance, addition of vitamin E in the diet has shown a beneficial effect in layers (31, 463), but other researches have found no effect with addition of this vitamin to the diet (447, 451). Some drugs, such as nicarbazin or monensin (404, 451), have deleterious interactions when administered to heat stressed birds; while others, such as virginiamycin (451), may alleviate heat distress.

Preventive measures consist of installation of fans and foggers, proper construction of ventilating ducts, insulation of the buildings and water pipes, roof overhangs to prevent the sunlight from shining into the house and use of white or aluminum paint on the outside to reflect heat. In southern climates where low production and mortality from heat are constant problems, installation of foggers and sprinklers or evaporative coolers is essential. Also, enhancement of adaptability of birds to heat stress conditions, such as early thermal conditioning should be considered (91).

Asphyxiation

Asphyxiation is generally caused by birds crowding or piling in a corner. It may occur when birds are moved to new quarters, when they are frightened, or in young birds when they are chilled. Asphyxiation also may happen when power fails or ventilation system is faulty in controlled-environment houses that have no windows. The history of the case often indicates that mortality occurs only at night and the flock in general looks healthy. Asphyxiation of baby chicks can occur in chick boxes that are piled too high without an air space between each box, in boxes that do not have sufficient ventilation holes, or in boxes placed in a closed compartment such as the trunk of a car. Necropsy of birds that have asphyxiated usually does not reveal specific gross or histologic lesions to make a positive diagnosis, but a thorough examination will eliminate other possible causes of death. In birds that have smothered, there is congestion of the trachea and lungs, and in older birds feathers will be worn off where birds have been trampled.

Asphyxiation of chicks in the brooder house can be controlled by putting a circle of corrugated cardboard around the hover for the first week of life and gradually widening the diameter as chicks get older. This will prevent piling in a corner during the night. When birds are moved to new quarters, the use of a dim light or lantern the first few nights will decrease the possibility of smothering. Birds transferred to new quarters should be checked late in the evening for signs of piling. Frequent observation of the flock is very important the first few days after acquiring a group of new chicks or grown birds.

Dehydration

Dehydration is generally caused by failure of birds to find water, inability to reach the water, failure to provide adequate amount of water, or in some cases by a deterring factor in the water. Chicks can survive several days without water but will die beginning on

the 4th or 5th day. Mortality will reach its peak during the 5th or 6th day and terminate abruptly if water is provided. Chicks that are not drinking will have succumbed by this period, and survivors are those that have found the water and are drinking. Laying birds need a constant water supply or production will drop. Birds will die if the water restriction is severe. Dehydration can be detected by the chick's inability to "peep" during the later stages, insufficient weight for size and age, and dehydrated and wrinkled skin on the shanks. Other changes are blue discoloration of the beak, dry and dark breast musculature, dark kidneys, accumulation of urates in the ureters, visceral urate deposition ("visceral gout"), and darkening of the blood. Signs and lesions in older birds are similar to those in chicks, and weight loss is much more noticeable. To prevent dehydration in chicks, water fountains should be placed at the edge of the hover directly on the litter without any platform. When a large type or automatic drinkers replace a small drinker, the old type should be kept for a few days and gradually moved toward the new source of water supply to accustom birds to the change. Faulty electrical heating devices used to prevent freezing may cause an electrical charge in the water, and birds will not drink.

Incubation and Hatchery Related Problems

It is a common misunderstanding in the poultry industry that health problems in birds begin with hatching. Incubation conditions have a profound effect on chicken survival and quality. Poor incubation causes major losses to the industry, not only due to low hatchability or increased early mortality (146); but also due to growth depression, unevenness of the flock, increased susceptibility to infectious agents, and increased incidence of leg problems (370). It is important to identify the problem in order to minimize the losses in a flock. Temperature, humidity, ventilation, and egg turning are critical factors for hatching good quality chickens and turkeys. Embryos or recently hatched birds are more susceptible to chilling or overheating than older birds. The most typical complaint caused by high incubation temperature, and sometimes from low humidity, is "unhealed navels." Unhealed navel is a broad term that includes black buttons, strings, and ectopic viscera. Temperatures of 3 degrees higher than normal during the last week of incubation are also associated with increased number of late dead embryos, unevenness in the flock, skin necrosis, and high incidence of leg problems including slipped tendons. Effects of low incubation temperature are more pronounced during the first week of incubation. Some of the effects of low temperature include high incidence of late dead, pipped alive, increased residual albumen, unsteady gait, and high incidence of spraddle leg deformities. Low temperature while in the hatcher induces increased number of navel abnormalities such as improper closure of the navel, bloody navels, and urates attached to the yolk sac. Starvation and dehydration may occur when birds are accidentally held in hatching machines for long periods.

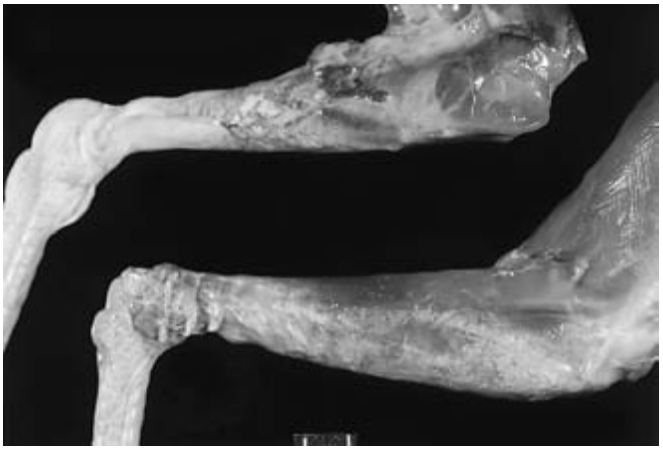
Early chick mortality has also been associated with rupture of the yolk sac. Late hatching chicks may have a higher incidence of ruptured yolk sac. Because of the dry conditions in the hatcher the yolk sac may adhere to the wall of the abdomen reducing its mobility and increasing its susceptibility to injury during hatchery manipulations. Also, during sexing the handler may squeeze the birds too firmly, increasing the incidence of ruptured yolk sacs (418). If the humidity during incubation is higher than normal, the hatchlings may have large yolk sacs. These yolk sacs are not well suited to withstand standard handling practices at the hatchery and consequently there is an increased incidence of yolk sac ruptures (268).

Prevention can be achieved by establishing standards for the hatchery and maintenance of equipment, especially ventilation systems. Environmental temperature, humidity and pressures in every room should be taken on a daily basis and variations in these parameters should be minimized. Diagnostic work of the unhatched eggs left in the hatching trays should be done to establish specific causes of mortality or poor performance in the flock and to solve hatching problems. Optimizing incubation conditions and handling birds with care during sexing can minimize the incidence of ruptured yolk sac (17, 268).

Starve-out

Mortality in young birds, between 1 and 10 days of age, that is not from infectious diseases is commonly referred to as starve-out because it is generally associated with lack of water and nutrient intake. Management and environmental factors including temperature, light, and water and feed quality may contribute to early mortality. Under commercial hatchery conditions, birds may be 24–48 hour old before they are removed from the hatchery, and additional time is spent in processing and transportation to the farm. As a result, most birds are delivered when they are 50 hours old or older, and will do well if they are placed in brooders and given adequate feed and water. However, mortality up to 6.14% has been reported in birds if they are placed at 72 hours after hatching. Mortality increased to as high as 35.14% if they were not placed until 120 hours after hatching (132). As soon as the birds are placed in the brooders, they should be encouraged to eat and drink. There are no specific gross lesions associated with starve-out. In general, the starve-out birds are smaller, dehydrated, and have dark shanks. The crop, proventriculus, and gizzard may contain litter material, but not feed. Infectious diseases should be ruled out before making a diagnosis of starve-out.

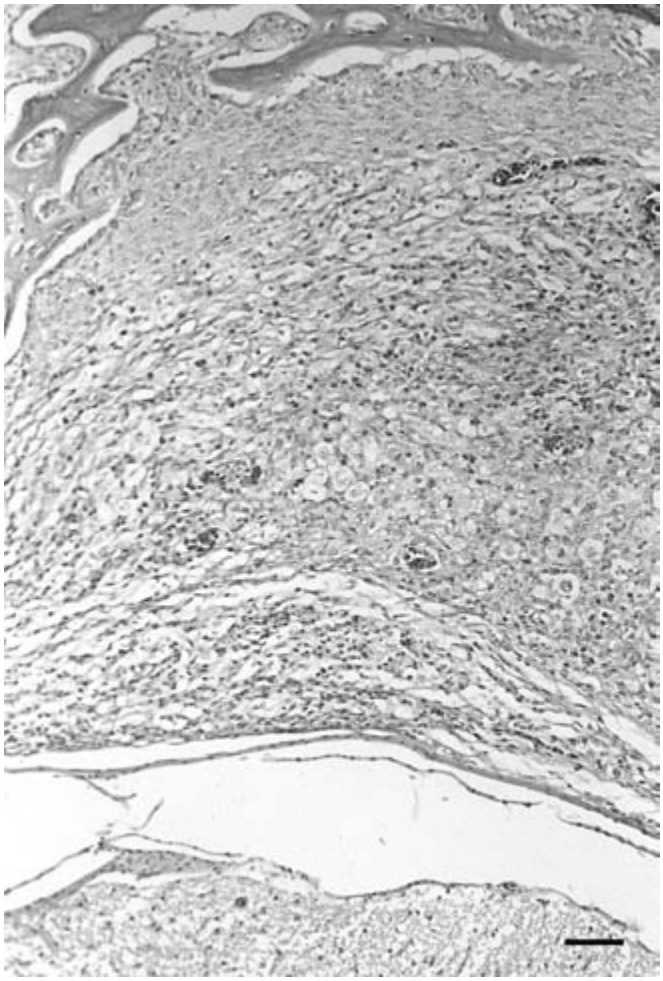
Management practices that delay or discourage birds from eating and drinking should be avoided in order to prevent starve-outs. The optimal temperature in the house at placement and during the first week is crucial for encouraging birds to seek for water and feed. The birds must have adequate light to easily find the feeders and drinkers. Drinkers and feeders should be adequately spaced and easily accessible. The feed should be palatable. If the crumbles or pellets are too large, the birds will not be able to eat them. If the feed is too fine, it will stick to the beaks of the birds. If the feed or water is too warm the chicks will not eat or drink. Some hatcheries inject the birds subcutaneously with a glucose solution to prevent starve-outs. However, no differences were found in the total mortality between fasted and glucose treated poults at 2 wk of age (304).



30.1. Layer chickens with severe myositis and muscle atrophy secondary to vaccination. Bar = 1 cm.

Problems Related with Vaccination

Killed vaccines and bacterins usually employ an oil adjuvant to stimulate a localized inflammatory reaction and improve the immune response. When oil-emulsion bacterins are administered subcutaneously into the neck, the adjuvant may infiltrate into the adjacent tissues causing dermatitis, neuritis, and myositis (2). Neurological and/or musculoskeletal problems, which impair the birds' ability to eat, have been documented when a killed vaccine is delivered subcutaneously in the neck and/or too close to the skull. Killed vaccines administered intramuscularly into the breast or leg muscles (Fig. 30.1) may produce severe granulomatous myositis near the site of injection (98). The birds may refuse moving due to muscle damage, lose weight and have reduced production; besides, the meat may be downgraded at the processing plant due to trimming of affected areas. Live or attenuated vaccines and bacterins also have been reported to cause pathologic changes in the birds. In young chickens, in ovo and subcutaneous live vaccines given at the hatchery have been associated with neurologic signs, pyogranulomatous myositis, neuritis, and meningomyelitis (Fig. 30.2). Since these vaccines do not have irritant substances, the tissue reaction is thought to be a misdirected vaccine (168, 424). Bacterial contamination of the hatchery's vaccine equipment has caused nervous symptoms and increased mortality in young birds (298). To minimize the inflammatory reaction in the tissues, manufacturer recommendation should be followed in vaccinating birds and vaccinating crew should be trained in injecting the bacterins and vaccines into the correct site. Vaccine equipment should be maintained clean and disinfected to avoid bacterial infections.



30.2. Histologic section of meninges around the spinal cord (bottom) of a 2-day-old broiler chicken that was vaccinated in the neck. Note the nonsuppurative meningitis, most likely associated with misdirected vaccination. H & E, bar = 10 μm

Amyloidosis

Amyloidosis is a well-recognized pathologic disorder in birds, characterized by deposition of proteinaceous material between cells in various tissues and organs of the body. Once the deposition of amyloid has started the progression of this disease is irreversible, because these proteins have low solubility and are relatively resistant to proteolytic digestion (239). There are more than 15 biochemical types of amyloid protein recognized in mammals (69); however, only amyloid A (AA) has been detected in birds (251, 310). Amyloidosis caused by AA is frequently associated to an underlying infectious or inflammatory condition (238). Landman *et al.* (251) have published an excellent review of amyloidosis in birds. Among the domestic avian species, waterfowl are most susceptible to amyloidosis. Birds of all ages are susceptible to amyloidosis, but it is most common in adults, although it can occur in ducks as young as 4 wk of age (420).

Clinical Signs and Pathology

No specific clinical signs or gross lesions are associated with systemic amyloidosis. Clinical signs in ducks may include anorexia, lethargy, loss of weight, decreased egg production in layers, swollen abdomen, and increased mortality in the flock. In Brown egg-laying type chickens locomotor problems due to

swollen joints and weight loss can be encountered. But often the birds with amyloidosis are submitted for necropsy after being found dead with no prior clinical signs.

Brown egg-laying type chickens are particularly susceptible to amyloid arthropathy caused by *Enterococcus faecalis* (252, 253) and by *Mycoplasma synoviae* (MS)(250). Amyloid arthropathy associated with MS has also been reported in turkeys (427), but the disease has not been reproduced experimentally. Other bacteria such as *E. coli, Salmonella enteritidis* and *Staphylococcus aureus* have also been associated with amyloidosis in chickens (249). The authors also have observed amyloidosis associated with *M. gallisepticum*. Another condition in which amyloidosis is frequently encountered in mature chickens is hepatitis-splenomegaly syndrome, which results from infection by hepatitis E virus (see "Hepatitis E Infections" in Chapter 14). Amyloidosis associated with mycobacteriosis is a common finding in waterfowl and other birds in zoological collections (164, 303). Management and genetic factors may also play an important role in the incidence of amyloidosis especially in ducks raised commercially.

Amyloid deposition may be found in any tissue; the most commonly affected organs are liver, spleen, intestine and kidney. Gross lesions may be none or minimal when amyloid deposits are present in small amounts. When present in significant amounts the lesions include severe ascites ("water belly") most common in ducks and diffusely enlarged heavy liver with firm to rubbery consistency and pale or brown or grey smooth surfaces (Fig. 30.3.). Cut surfaces of the liver may have a smooth waxy appearance and the capsule may be thickened due to fibrosis. Occasionally the livers of some affected birds may have multiple hyperplastic nodules of various sizes that may have the normal color of the liver. Spleen can be severely enlarged and mottled white (Fig. 30.4). Kidneys and adrenals may be enlarged and pale. Brown egg-laying type chickens with amyloid arthropathy may have enlarged joints with presence of orange-yellowish material in the joints (Fig. 30.5).

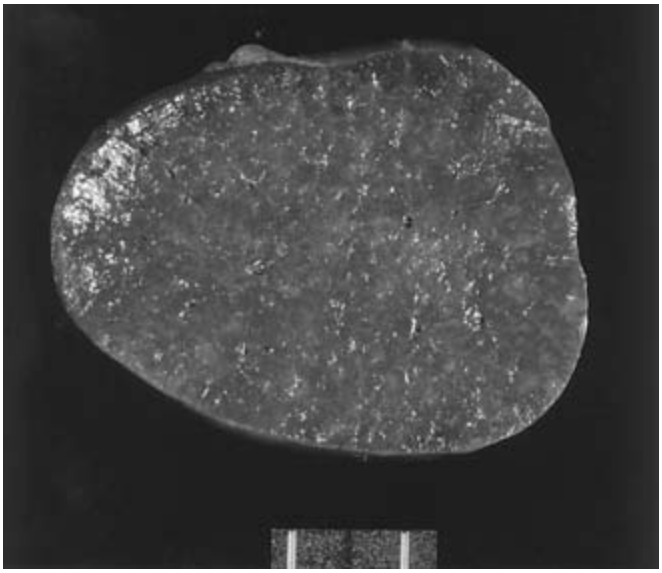
During the gross examination, application of Lugol's iodine to a suspected organ brings out the amyloid as distinctly brown material. If this is followed by application of dilute sulfuric acid, amyloid will change from brown to blue. Microscopically, amyloid appears as homogenous eosinophilic material deposited extracellularly in many organs (Fig. 30.6a). When amyloid is stained with Congo red it appears as brownish-orange and emits apple green birefringence under polarized light (Fig. 30.6b and 30.6c). Amyloid A can also be extracted and identified from formalin-fixed tissues using the Shtasrburg method (433).

Treatment and Prevention

There is no treatment for amyloidosis. Adequate management to prevent chronic infections or stress in birds would reduce the incidence of amyloidosis. Deposition of amyloid interferes with the normal functions of the affected tissue and it can be life threatening. Thus, treatment of the underlying inflammatory disease or stress factor should start as soon as possible to prevent or stop the progressive deposit of amyloid in the tissues. In a recent study, deposition of AA in the joints of chickens was enhanced by feeding a high dose vitamin A, while methylpred-



30.3. Hepatic amyloidosis in a 57-wk-old duck. The liver is severely enlarged and pale. Note the impression of the rib cage on the right lobe.



30.4. Splenomegaly due to amyloidosis in a duck. Multifocal white foci are throughout the spleen. Bar = 1 cm.

nisolone (an antiinflammatory drug) and pentoxifylline had an inhibitory effect (415).

Diseases of the Skeleton

Dyschondroplasia

Dyschondroplasia is restricted to the abnormal persisting accumulation of cartilage from the growth plate in which maturation of prehypertrophic and hypertrophic chondrocytes is retarded or stunted. It is a common defect associated with the growth plates of meat-type chickens, ducks, and turkeys. It is characterized by an avascular plug of abnormal cartilage in the growth plate of long bones. It is most commonly recognized in the proximal tibiotarsus, and, hence, the condition is often described as tibial dyschondroplasia. Dyschondroplasia also occurs, but is less severe, in the proximal and distal femur, the distal tibia, the proximal tarsometatarsus, and the proximal humerus (363). Reviews on the condition have been written by Farquharson and Jefferies (134), Orth and Cook (338), Thorp (454), and Whitehead (474).

Clinical Signs and Pathology

In many broiler chicken flocks up to 30% of birds may have lesions of dyschondroplasia characterized by abnormal masses of cartilage below the growth plate, primarily in the proximal tibiotarsus (Fig. 30.7), but also at other sites. In turkey flocks the incidence of dyschondroplasia has been reported as high as 79% (470). Most birds show no clinical signs. If masses of cartilage are large, birds may exhibit reluctance to move, stilted gait, and bilateral swelling of the femoral-tibial joints often associated with bowing of the legs. The abnormal masses of cartilage in the proximal tibia tend to be cone shaped. In mild cases, these cones of abnormal cartilage mainly develop below the posterior medial part of the growth plate. In severe cases, masses of cartilage develop from the whole growth plate and fill the whole metaphysis. Severity of the tibial dyschondroplasia lesions has been correlated with the degree of anterior bowing of the tibiotarsus and lameness in broiler chickens (272). The concave surfaces of such bones have hypertrophied cortices. This is considered an adaptive change (122). A high association between leg deformities and tibial dyschondroplasia also has been described in turkeys (470). Fractured fibulae have been associated with tibial dyschondroplasia and bowing of the tibia (363). These fractures occur at the iliofibularus, but are not always associated with abnormal tibiotarsal curvature (106). Dyschondroplasia in the femoral head in broiler chickens has been associated with a widened and shortened femoral neck and, in some cases, with fractures of the femoral head (99–101).

In a survey of leg weakness in broiler chicken flocks processed at 7 wk of age or earlier, few birds were culled because of dyschondroplasia (399). However, dyschondroplastic lesions can be recognized on radiographs and with a hand-held lixiscope from 2 wk of age (454). Interestingly, dyschondroplasia within the proximal tibiotarsus appears to be bilateral, and the incidence and severity of the tibial dyschondroplasia is similar in both legs (134). Downgrading of carcasses and trimming of deformed legs at processing have been attributed to dyschondroplasia (48, 399).



30.7. Medial view of sagittal sections of two proximal tibiotarsal bones from broiler chickens with tibial dyschondroplasia. The abnormal cartilage is only present in the posterior part of the metaphysis (*right*); abnormal cartilage fills the whole metaphysis, and the proximal end of the bone is enlarged (*left*). (Craig Riddell)

If broiler chickens are kept to roaster weights, lesions due to dyschondroplasia may be much more severe. In such birds, fractures below the abnormal cartilage in the tibia may cause severe crippling (Fig. 30.8). Resolution of the abnormal cartilage may start as early as 48 days of age, but sequestra of abnormal cartilage separated from the growth plate and bowing of the tibia may persist to as late as 30 wk of age, even though the proximal growth plate of the tibiotarsus in a chicken closes at 16–17 wk of age.

In turkeys, dyschondroplasia is recognized as early as 5 weeks (363). It peaks between 12 and 14 weeks of age (192). The incidence rate of dyschondroplasia start dropping around 15 wk of age, until the age of closure of the proximal tibial physis at 22 to 24 weeks but 5% or more of the toms may still retain some abnormal cartilage (470). Whereas there is no correlation between the body weight of turkeys and dyschondroplasia in early ages, the severity of dyschondroplasia is directly correlated with body weight between 14 and 15 wk of age (375).

Microscopically, dyschondroplasia is characterized by persistence and accumulation of prehypertrophic cartilage. The separation of the prehypertrophic cartilage from the proliferating cartilage is not sharply demarcated and few vessels penetrate the abnormal cartilage from the metaphysis. Normal growth plates or those with small dyschondroplastic lesions have few or no apoptotic chondrocytes, while in severe lesions numerous apoptotic cells with shrunken nuclei and little cytoplasm are present (378), suggesting that apoptosis is secondary to the formation of the car-



30.8. Sagittal sections of tibiotarsal bones from a roaster chicken with tibial dyschondroplasia. The severe angulation of the bones is due to fractures below the abnormal cartilage. (Craig Riddell)

tilaginous plug. Other studies have shown quite the opposite, that the dyschondroplasia lesions are associated with lack of apoptosis, which may be responsible for the retention of chondrocytes (326).

Ultrastructural and biochemical studies have demonstrated that the lesion begins in the prehypertrophic zone. The chondrocytes in the abnormal cartilage do not differentiate into fully hypertrophic chondrocytes (173, 362, 473), which are needed for cartilage vascularization, mineralization, and resorption. The abnormal chondrocyte mitochondria retain less calcium and phosphorus as compared to normal chondrocytes. Changes in extracellular matrix composition between the abnormal cartilage and normal hypertrophic cartilage that have been reported include lower levels of sulfur, potassium, calmodulin, alkaline phosphatase, collagen type X, prostaglandin precursors, proteoglycans, glycosaminoglycans, and transforming growth factor-ß (TGF-ß) and c-myc protein (133, 270, 338, 454, 473). The increased in nonreducible collagen cross-links observed in the matrix of abnormal cartilage in chickens (338) may be associated with the lack of release of collagen type X from the chondrocytes (473).

Pathogenesis and Etiology

The pathogenesis of dyschondroplasia is not well understood. Many hypotheses associated with defects of metaphyseal vasculature tried to explain the cause of dyschondroplasia. These include abnormal cartilage that cannot be invaded by metaphyseal vessels (363), occlusion of vascular canals from the epiphysis (101) and defective degradation of cartilage (256). It is now accepted that dyschondroplasia is a consequence of an inability of the prehypertrophic chondrocytes to undergo terminal differentiation. It is essential to recognize the mechanisms involved in the maturation of chondrocytes in order to prevent dyschondroplasia; unfortunately, these processes are not fully understood.

Some studies have shown that locally produced peptide growth factors play important autocrine and paracrine roles in development of the growth plate (261). A malfunction of one of these factors may be important in the development of tibial dyschondroplasia (454). TGF-ß has been found in the prehypertrophic and hypertrophic chondrocytes of the growth plate, and it regulates chondrocyte differentiation (457). TGF-ß expression was reduced in transitional chondrocytes in tibial dyschondroplasia, but its expression was increased where the lesion was being repaired (270). Fibroblast growth factor-ß, a potent angiogenic factor, is also reduced in tibial dyschondroplasia (487). Additionally insulin-like growth factor-I (IGF-I), basic fibroblast growth factor, and transforming growth factor-α, which are normally found in the more mature chondrocytes of the growth plate, are reduced in dyschondroplastic chondrocytes (457). Systemic as well as local growth factors may be important in the pathogenesis of tibial dyschondroplasia (456, 457). An altered paracrine function of growth plate "macrophages" may result in reduced cartilage degradation and tibial dyschondroplasia (68). Alkaline phosphatase and type X collagen, markers of chondrocyte differentiation, as well as c-myc protein an inducer of apoptosis, are reduced in the extracellular matrix of dyschondroplastic lesions (134, 473). On the other hand, the chondrocytes still retain the ability to express alkaline phosphatase and type X collagen (379, 473). This suggests that tibial dyschondroplasia is due to a metabolic dysfunction rather than to an alteration in the expression of genes.

Vitamin D_3 and its metabolites have been shown to reduce dyschondroplasia. A recent study (477) showed high concentration of vitamin D_3 (250 µg/kg) prevented tibial dyschondroplasia. Previous to this report, only addition of vitamin D metabolites, such as 1,25-dihydroxicholecalciferol, have been shown to be very effective in reducing the incidence of dyschondroplasia (134, 382). No correlation between the plasma level of 1,25-hydroxycholecalciferol and the incidence of tibial dyschondroplasia was found in one series of experiments, and it was suggested that the ability to utilize the vitamin D metabolite at the receptor level might affect the incidence of tibial dyschondroplasia (129). On the other hand, Parkinson *et al.* (348) found some broiler strains that have a higher predisposition to dyschondroplasia also have lower serum concentrations of 1,25-hydroxycholecalciferol. However the higher concentration also decreased body weight. The incidence of tibial dyschondroplasia was prevented when chicks received ultraviolet radiation even without vitamin D_3 supplementation (125).

The incidence and severity of tibial dyschondroplasia can be influenced by nutrition and genetic selection (134). Rapeseed meal, sorghum, and some types of soybean have been suggested to increase the occurrence of tibial dyschondroplasia (469). Some dietary supplements that are linked to increased incidence of dyschondroplasia include rations containing added cysteine or homocysteine (338), copper-deficient diets (260), rations contaminated with the fungus *Fusarium* sp. or its product fusarochromanone (59, 338), and rations containing dithiocarbamate fungicides (338, 377, 379). Also some antibiotics, such as zinc bacitracin and salinomycin, may increase the incidence of tibial dyschondroplasia (469).

Though in original reports calcium and phosphorus levels in the ration were considered to have no effect, it has been shown that the incidence and severity of tibial dyschondroplasia in broiler chickens can be increased by feeding high levels of phosphorus relative to the level of calcium (124, 127, 398, 400). Feeding turkeys similar levels of phosphorus and calcium did not result in a high incidence of tibial dyschondroplasia in turkey poults (405). Tibial dyschondroplasia in broiler chickens was not eliminated by feeding a ration containing 1.5% calcium and 0.5% available phosphorus (398).

It has been assumed that rapid growth is a major cause of tibial dyschondroplasia, because restricting feeding reduces the incidence of this disorder (400). The presence of the most severe lesions of dyschondroplasia in the proximal tibiotarsus may be due to the growth plate at that site having the most rapid growth. Daily fasting can also reduce incidence of tibial dyschondroplasia without causing growth depression (126). It has been suggested that diurnal rhythms may be important in reducing tibial dyschondroplasia (129). An interrupted- and increasing-light program had no effect on clinical and subclinical tibial dyschondroplasia in roaster chickens (394). An intermittent light program reduced the incidence of tibial dyschondroplasia in one line of broiler chickens, but not in another two lines (506). Although reducing the growth rate of experimental birds decreased the incidence of tibial dyschondroplasia, there was no direct correlation between growth of individual birds and the incidence of tibial dyschondroplasia (246, 388). Numerous investigators (386, 455, 505) have shown that the occurrence of tibial dyschondroplasia is susceptible to genetic selection. After several generations of selection Yalcin *et al.* (507) were able to reduce the incidence of tibial dyschondroplasia without affecting the body weight of the birds.

Osteoporosis

Osteoporosis in laying hens is defined as a decrease of normal mineralization of structural bone, resulting in increased fragility and susceptibility to fracture. It was first described in caged laying hens that had brittle bones and were unable to stand, but willing to eat and drink (70). The condition was then called "cage layer fatigue." Osteoporosis is still the most significant disease of the skeleton of laying hens kept in battery cages. Bone fragility is responsible of up to 30% of fractures of commercial flocks during their life, and the incidence may reach up to 90% during catching, transporting, and processing (162, 476).

Clinical Signs and Pathology

Osteoporosis normally consists of loss in bone quality which predisposes the birds to fractures in a range of bones of the body. Ischium, humerus, and keel bones show the highest incidence of fractures, followed by fractures of pubis, ulna, coracoid and femur (160). In the most severe cases, it can cause paralysis due to the collapse of the spinal bone (16). They often are alert, but later become depressed and die from dehydration. Osteoporosis is more severe between 25 and 50 wk of age (138).

On postmortem examination, fractures are normally found in leg and wing bones, and bones throughout the skeleton are easily broken. If the bird is paralyzed, fractures of the vertebra are rarely seen; however, the loss of structural bone may lead to exposure of the spinal cord leading to pressure on exposed nerves (476). Sterna are often deformed, and there is characteristic infolding deformation of the ribs at the junction of the sternal and vertebral components. The bone cortex is thin, but there are no changes in the external dimensions of the bone, because cortical bone resorption is restricted to the endosteal surface (11). Parathyroid glands are enlarged. Many birds have regressive ovaries and are dehydrated, while some dead birds have an egg in the oviduct and have died acutely.

On histology, the cortices of bones are thin, with enlarged absorption spaces. Medullary bone is reduced in amount, and largely consists of osteoid. The deformation of the ribs can be due to small fractures. Damage to the spinal cord is often associated with pressure on the nerves, which may cause paralysis.

Pathogenesis, Etiology, and Control

With the onset of sexual maturity there is a rise in estrogen that results in a switch from structural bone formation to accumulation of medullary bone (476). The resorption of structural bone starts at sexual maturity and continues during the production life so that osteoporosis is more severe at the end of lay. Additionally increased bone fragility is associated not only with loss of mineral, but also with modifications in the collagen structure (240, 440).

Type of housing has been shown to affect the incidence of osteoporosis. Confinement of laying hens in cages has been shown to reduce bone strength significantly (241, 323). Structural bone loss and bone strength are directly associated with the amount of exercise allowed. Numerous publications discuss the relationship between type of housing and bone strength (139, 265, 472, 475). Bones from laying hens kept in perchery systems are stronger than those from hens kept in enriched cages, litter or wire systems; while hens kept in conventional cages had the weakest bones. One experiment demonstrated that bones from egg layers became stronger after just 20 days of transferring the birds from cages to floor pens (320). Studies directed at defining and preventing bone breakage when hens are processed at the end of their production cycle revealed that a greater incidence of freshly broken bones in laying hens after handling at processing time has been described in hens from cages, compared with hens from other housing systems (162). The incidence of bone fractures is also influenced by the method of handling (161).

Nutritional methods of preventing osteoporosis have not been successful. However poor nutrition may exacerbate the problem. The formation of strong cortical bone and adequate medullary bone prior to egg production may be helpful in reducing osteoporosis. Increased calcium in the ration prior to egg production may be necessary, but it has been suggested that if increased calcium is fed for too long before egg production, the parathyroid gland may be suppressed. Feeding calcium in particulates, either as oystershell or limestone granules, may extend the period of calcium absorption during the night, which reduces the depletion of medullary bone and benefits the eggshell quality (137, 138); however it does not have much impact on the loss of structural

bone. Combination of limestone with fluoride and/or vitamin K_3 during the laying period had no greater benefits than limestone alone (137).

Treatment of pullets with alendronate, an inhibitor of bone resorption, just prior to the onset of lay decreased the loss of cancellous bone (460). Unfortunately at the time of lay, alendronate prevented only the loss of medullary bone, but did not stop the loss of structural bone (500).

Selection pressure to maximize egg production might have contributed to the osteoporosis by producing strains of birds with poor bone quality. Bishop *et al.* (24) were able to reduce the incidence of bone fractures and increase bone strength after five generations in a commercial line of white leghorns. They found that cancellous and medullary bone volumes were poorly heritable parameters; but keel radiography density, humeral and tibial strengths, and index calculated from these 3 traits are promising predictors of bone characteristics and resistance to osteoporosis.

Valgus and Varus Deformation

Deformation of the long bones of the broiler chicken and turkey is a significant cause of economic loss due to culling and death of affected birds. Such deformation includes many different types of twisting or bending of the bones and has been described by terms such as *long bone distortion*, *twisted legs*, or *crooked legs*. The general topic of deformation of the long bones in domestic poultry was reviewed by Riddell (388, 391) and Thorp (453, 454). The most common type of long bone deformation in the broiler chicken is valgus or varus deformation (VVD) of the intertarsal joint (219, 373, 399). In the turkey, similar deformation of the intertarsal joint is also common but is often associated with varus deformation of the femoral-tibial joint (387). In broiler chickens, the incidence of birds affected with varus deformation varies from 1 to 3% while valgus of the tarsal joint affects 30 to 40% of the birds (263). Male broilers have higher incidence of VVD than females (219).

Clinical Signs and Pathology

Valgus or "knock-kneed" results from the outward deviation of the tarsometatarsus when placed in line with the tibiotarsus (373). Varus deviation or "bow-legged" is the consequence of inward deviation of the tarsometatarsus when lined with the tibiotarsus (373). The major deformity is in the distal tibiotarsus, with similar but less severe angulation in the proximal tarsometatarsus (Fig. 30.9 and 30.10). The valgus deviation is more common; but varus deviation may result in more restricted walking ability in poultry (67, 263). The defect may affect both legs but is often unilateral, with the right leg more commonly affected than the left leg (121, 399). Approximately 70% of affected birds are males (399). Most birds have either valgus or varus deformation, but the occasional bird will have valgus deformation of one leg and varus deformation of the other leg. These birds have been described as "windswept" (112). Abnormal rotation of the femur may also be present (121).

Leterrier and Nys (263) observed that valgus angulation appears progressively between 2 to 7 wk of age and is often bilateral; on the other hand, varus deviation appears suddenly between 5 and 15 days of age and most of the cases are unilateral. Other researchers found a similar appearance and progression of both defects with age (219, 389). As the severity of the valgus angulation increases, the gastrocnemius tendon may become displaced and the distal tibial condyles become flattened. In the varus de-



30.9. Broiler chicken with unilateral valgus deformation of the intertarsal joint. (*Avian Diseases*)



30.10. Tibiotarsal and tarsometatarsal bones from a broiler chicken with unilateral valgus deformation. (*Avian Diseases*)

viation, the gastrocnemius tendon is always displaced medially (263). In some cases, the angulation progresses to displacement and separation of the tarsal bones from the shaft of the tibia. With severe angulation, birds are forced to walk on the posterior surface of the hock, which becomes bruised and swollen. In some instances, the distal shaft of the tibia will penetrate the skin.

Pathogenesis and Etiology

The pathogenesis of the deformation has not been defined. The last 40 years have seen an increase in growth rate as well as incidence of VVD. Slowing growth rate can reduce the incidence of VVD (188, 198, 389). However, reducing energy in the diet does not improve the quality of the cortical bone and reduced limb angulation might be due to lower body weight placing less stress on the skeleton (264). A higher incidence of VVD occurs in broiler chickens raised in cages compared with broiler chickens raised in floor pens (188, 380, 389). This may be explained by a lack of exercise in cages (188). Stronger cortical bone in chickens occurs with exercise (402). Different photoperiods will affect the incidence of VVD (63, 394). It is unknown whether this is due to a change in growth rate, amount of exercise or a hormonal factor. An increased incidence of angular limb deformities has been reported in turkeys subsequent to malabsorption syndrome at an early age (358).

Young normal broiler chickens have slight valgus deformation. This small inclination of the growth plate in a rapidly growing chicken may promote deviant growth (373, 396). In the rapidly growing modern broiler chicken, the vascular morphology of the growth plate is irregular, and this may predispose the chicken to VVD (452). Some workers (88, 205, 206) have noted a delay in cortical bone differentiation, which precedes the angulation. It has been suggested that valgus and varus deformities may each have a different etiologic pathogenesis (263) and that these deformities may be heritable (258). It is also considered that selection may influence the incidence of leg deviations. Le Bihan-Duval *et al.* (259) estimated that the susceptibility to valgus deformity was genetically independent of meat conformation, while varus deformation increased with body weight.

VVD should not be mistaken with leg deformities caused by several nutritional deficiencies, such as manganese deficiency. In manganese deficiency there is a generalized disorder of the growth plates or dyschondroplasia of the long bones (388). No evidence of the microscopic growth plate lesions has been recognized in VVD (373, 395). The possibility that submicroscopic lesions of the growth plate due to marginal nutritional deficiencies may result in VVD should not be ignored. An association between VVD and dyschondroplasia has been noted (363, 373, 389). Although dyschondroplasia may weaken bones and predispose to deformation, it may be secondary to the deformation (502). In a breeding study, it was observed that VVD was unrelated to dyschondroplasia (386).

Degenerative Joint Disease

Degenerative changes in joints have been recognized primarily in coxofemoral joints of mature male turkeys (103, 107) and mature meat-type chickens (107, 113), and in the spine of laying hens (508). They have also been reported in the femoral-tibial and intertarsal joints of turkeys (104, 118) and male broiler breeding fowls (113, 117). Duff (103) reviewed early reports of degenerative hip disease in poultry.



30.11. Anterior and lateral views of distal femora from breeding turkeys with degenerative joint disease. Note the erosions, and thinning of the articular cartilage.

Clinical Signs and Pathology

Degeneration of the articular cartilage causes pain and lameness (123) by exposing the subchondral bone and impairing the ability of the cartilage to provide a smooth articulating surface to the joint (65). Turkeys with degenerative hip disease assume a stance with abducted pelvic limbs and constantly shift weight from one leg to another and are reluctant to move. In turkeys, the initial gross lesions are found in the articular surface of the antitrochanteric region of the acetabulum (103). As the degeneration progresses both the femoral and acetabular components of the coxofemoral joint become involved. Degenerative lesions are also found in the femoral-tibial and intertarsal joints (Fig. 30.11). Erosions, fissures and thinning are characteristic lesions of the articular cartilage. In addition flaps of cartilage and/or osteophytes that may break free to give fragments within the joint may form in the degenerative articular cartilage. The articular cartilage of birds with degenerative joint disease is more hydrated with a higher uronic acid content than those from normal joints (6). Periarticular fibrosis accompanies severe lesions (103).

Microscopic changes in the cartilage include loss of normal structure, areas of necrosis, fissures, and massive chondrocyte clusters (102). Surface fibrillation occurs in the articular cartilage (6). In some cases there is evidence of disturbed endochondral ossification. It was proposed that the lesion in such cases could be described as osteochondrosis dissecans (104). There is also mild hyperplasia and hypertrophy of the synovium (6).

Pathogenesis and Etiology

The pathogenesis of many of the joint lesions described above is not clear; however morphologic and biochemical changes of the articular cartilage are similar to mammals. Many may result from primary damage to the articular cartilage, while others may be sequelae to osteochondrosis (107, 113). Since heavy breeds, white-broad breasted turkeys and broiler chickens are more af-

fected by this disease, genetic factors may play a role in modifying cartilage development (6).

Spontaneous bone fractures

Bone fractures are one of the causes downgrading and trimming of poultry carcasses. Breakage of leg bones are economically more important. Fractures may occur spontaneously on the farm or during catching or transportation. In meat-type poultry, spontaneous fractures occur more frequently during the last part of the grow-out. In turkey breeders, complete fractures have been associated with preexisting stress or partial fractures in males (77). In laying hens, osteoporosis is the most common predisposing factor to bone fracture (see Osteoporosis section).

Clinical Signs and Pathology

Fractures of leg bones cause lameness in affected birds. Down birds may die if they do not reach feed or water or are killed by other birds in the flock. Femoral fractures were associated with increased mortality of up to 1% per week in flocks of heavy tom turkeys (77, 360). At necropsy birds had complete closed oblique fracture of the femur. In breeding turkeys the fracture line was associated with a site of callus formation (77). Lower ash, calcium and phosphorus were found in the bone cortex of broilers with fractures than those bones of birds from the same flock that did not have fractures (458). Similar findings have been reported in turkey breeder flocks (76).

Pathogenesis and Etiology

Poor bone quality increases the risk of bone breakage (78, 376). Growth rate is considered an important determinant of bone strength. One study (264) showed no improvement in mineral quality of the cortical bone when the growth rate was reduced; but this study did not investigate the effect on the organic components or the mechanical characteristics of the bone. Selection for increased body weight associated with increased breast muscle and no appreciable changes in the skeleton (67, 267) puts higher stress on the cortical bone increasing the probability of fractures. Heavy birds with large breast muscle are less active than light birds (190, 267); lack of exercise may be responsible for decrease of bone density (322). Growing male birds may be more susceptible to fractures than females, because males have a more porous cortex and less adapted to physical stress females (403). In turkeys, leg fractures occur more frequently in the femur of growing birds (267). There is indication that maturation, including mineralization rates, occurs slower in the femur than what is observed in the tibia (267). Bone strength is related also to the organic matrix composition, and especially to the collagen crosslinks of the cortex (374). Nutrition is also an important factor to bone strength. Imbalances in inorganic and/or organic nutrients may decrease the bone strength and increases the risk of fracture (376).

Handling of birds may create unnatural tensile and bending stresses on the bone, which may alter the cortex composition, predisposing the bones to complete fractures (77). To prevent and reduce incidence of fractures, care should be taken when catching birds. Chickens that are held by both legs have fewer incidences of broken bones than when they are held by one leg only (161).



30.12. Broiler chicken with spondylolisthesis. (*Avian Diseases*)

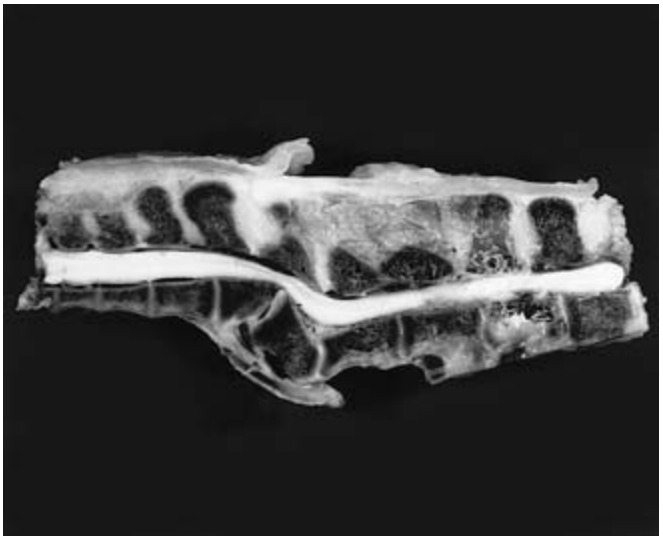
Spondylolisthesis

Spondylolisthesis, also known as "Kinky Back," is the ventral dislocation of the anterior end of the articulating fourth thoracic vertebra with over-riding of the posterior end by the fifth causing spinal cord compression and posterior paralysis in broiler chickens. This rotation causes a kyphotic angulation of the floor of the spinal canal and spinal cord compression (Fig. 30.12). The deformation of the spinal column can be readily recognized by palpating the ventral surface of the spinal column during necropsy. Another form of spondylolisthesis is characterized by steplike defects between adjacent thoracic vertebrae producing stenosis of the vertebral canal (115). A diagnosis of spondylolisthesis is best confirmed by removing, decalcifying, and splitting the spinal column along a midline longitudinal plane to allow visualization of the spinal cord compression. A few birds affected with spondylolisthesis are found in most broiler flocks. In some flocks, the incidence of affected birds has reached 2%. The peak incidence occurs at 3–6 wk of age. Affected birds are alert, remain sitting on their hocks with their feet slightly raised off the ground (Fig. 30.13), and use their wings in an attempt to escape when approached. Severely affected birds often become laterally recumbent. Affected birds often die from dehydration if not culled. Wise (501) and Riddell (388) have written reviews on the condition.

Lordosis and subclinical spondylolisthesis are common in broiler chickens. The lordosis develops after hatching. It can be decreased by slowing the growth rate of the broiler chicken. The incidence of spondylolisthesis can be increased by genetic selection. It is postulated that spondylolisthesis is a development disorder influenced by conformation and growth rate.

Ligament Failure and Avulsion

Lesions of ligaments of the intertarsal joint were first described as a significant cause of lameness in meat-type poultry by Craig (72). Lesions have since been reported in the capital femoral ligament in young adult broiler chickens (108), in the posterior cruciate and other ligaments of the femoral-tibial joint in young



30.13. Midline longitudinal section through thoracic-lumbar region of the spinal column of a broiler chicken with spondylolisthesis, cervical end to right. Rotation of the body of vertebra T4, deformation of T5, and spinal cord compression. (*Avian Diseases*)

adult broiler chickens (105, 109, 110, 117) and turkeys (103, 118), in the intercondylar and collateral ligaments of the intertarsal joint of turkeys (118, 228) and broiler chickens (111, 117). Avulsion of the retinaculum on the distal tibia of turkeys has been reported recently (75).

Clinical Signs and Pathology

Lameness has been attributed to lesions in the capital femoral ligament. Lesions found include stretching, partial or total rupture, and avulsion—sometimes with a piece of cartilage or bone—from the femoral head insertion. Stretched ligaments sometimes contain hematomas or are infiltrated with fat. Microscopic lesions include fraying of collagen bundles, acellularity and hyalinization of the collagen in the tendon, along with necrosis, fissures, and hemorrhage in cartilage adjacent to the site of insertion (108). Lameness has also been associated with lesions in ligaments of the femoral-tibial joint. The posterior cruciate ligament has been the most commonly affected, but the cranial cruciate, collateral, and caudal meniscofemoral ligaments have also been affected. In the cruciate ligament, total or partial rupture near the tibial insertion or avulsion from the tibial insertion occurs. Microscopic lesions are similar to those described for affected femoral capital ligaments. In addition, multicellular clusters and mucoid degeneration in the tendons and disorganization of subchondral bone with cysts and granulation at the avulsion site are found (105). Some abnormalities of the menisci of the knee joint have been associated with ligament disruption (114). Lameness has also been associated with partial or total rupture of intercondylar ligament and with rupture or avulsion of collateral ligaments. Most microscopic changes in affected ligaments have been similar to those described for other affected ligaments (111, 228). In contrast to the previously described clinical signs associated with avulsion of other ligaments, lameness was not observed in the affected birds due to avulsion of the retinaculum; however, there were increased condemnations of legs due to hematomas and muscle discoloration at slaughter (75).

Pathogenesis and Etiology

Ligament rupture is probably due to trauma. Microscopic lesions similar to these described in ruptured ligaments have been described in intact ligaments of broiler-type chickens, indicating that these changes precede the rupture (109). In individual male broiler breeding chickens, tendon or ligament failure is often found at more than one site, suggesting a predisposition to ligament and tendon failure in these birds (110). Ligament failure may in part be age related, as the incidence appears to increase with age (117). Ligament lesions were less severe in turkeys fed a restricted amount of feed when compared with turkeys fed *ad libitum* (118). Rupture of ligaments may be secondary to stress induced by limb angulation (111). In converse, it has been suggested that ligament rupture may result in limb angulation (228, 388).

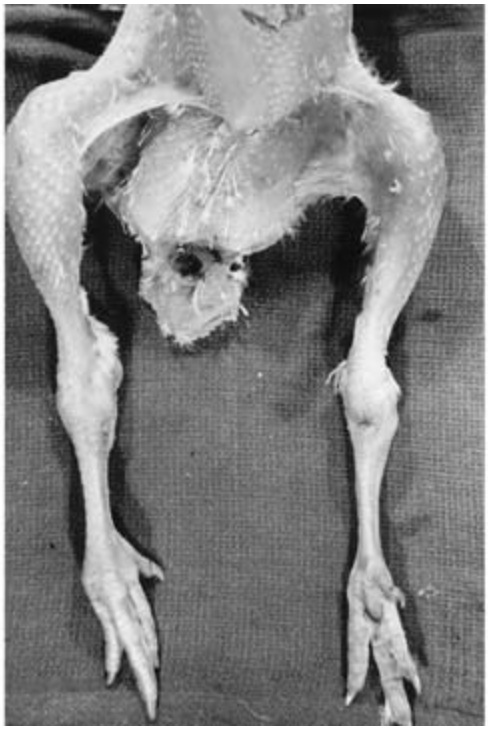
Other Abnormalities of the Skeleton

Osteochondrosis

Osteochondrosis is a focal degenerative lesion of the growth plate, articular cartilage or bone, associated with ischemic and necrotic lesions of the growth plate, articular cartilage or bone. It is rare in poultry, and when it occurs, its causes are usually related to focal bacterial infection (459) or mechanical forces acting on rapidly growing cartilage (120, 291). A variety of microscopic degenerate lesions including eosinophilic streaks or scars, occlusion and thrombosis of vascular canals, and necrosis in the growth plate and epiphysis have been described in growing meat-type birds (291, 396). These lesions may be associated with dyschondroplasia, and in some cases, may cause dyschondroplasia (101). Osteochondrosis has primarily been described in cervical and thoracic vertebrae of broiler chickens (201, 274), and in the femoral head (99, 101, 120, 229, 396) and the antitrochanter (107) of broiler chickens and turkeys. Most birds with osteochondrosis do not have clinical sings (291, 396).

Rotated Tibia

Rotated tibia has been reported in turkeys, broilers, guinea fowl, and ratites (388, 399, 446). Rotated tibia is very common in ratites especially in ostriches and emus. Affected birds often have the affected leg extended laterally. Either or both legs may be affected. The defect is restricted to the shaft of the tibia, which is rotated externally often to 90 degrees or greater. There is no angulation of bones, and the hock joint is normal, with no displacement of the gastrocnemius tendon. In some extreme cases, the rotation reaches 180 degrees. In such cases, if both legs are extended ventrally, the two-foot pads face in opposite directions (Fig. 30.14). Rotation or torsion of the femur, tibiotarsus, and tarsometatarsus is normal during early development of the chicken. Femurs and tibiotarsi rotate externally, while the tarsometatarsi rotate medially when the axis of the distal articular surface is compared with that of the proximal articular surface (122).



30.14. Turkey with rotated tibia of left leg. Rotation is nearly 180 degrees and the footpads face in opposite directions. (Craig Riddell)

Rotated tibia represents excessive and abnormal rotation during development. The exact etiology of tibial rotation is not known, but genetics, nutritional, and management factors may be involved (446). Early rickets has been suggested as a predisposing factor in guinea fowl (18). An increased incidence of rotated tibia has been reported in turkeys subsequent to malabsorption syndrome at an early age (358). Rotated tibia differs from VVD in that no angulation of bones is present, and that in broiler flocks, the peak incidence occurs at 3 wk of age, no sex predisposition is apparent, and the number of birds with either the right leg or left leg affected is approximately equal (399).

Crooked Toes

Crooked toes are a common finding in meat-type chickens and turkeys. The syndrome was first described by Norris *et al.* (324) and later reviewed by Riddell (386). An incidence of 4.8–7.7% was reported in broiler chickens in Europe (363), while incidences exceeding 50% were reported in broiler chickens in Australia (308). The deformity, unless very severe, has limited clinical significance. It may interfere with the reproductive performance of breeding cockerels (308). Most digits or a single digit may be bent laterally or medially. Rotation of the phalanges often is present.

The pathogenesis is not understood though it has been proposed that shortening of flexor tendons may cause the deformation. This defect also has been attributed to a hereditary defect (58). A negative correlation between the presence of twisted legs and slipped tendons and the presence of crooked toes has been reported. The tension in flexor tendons is probably decreased when legs are twisted (365). An increased incidence of crooked toes has been associated with certain types of flooring, infrared brooding, pyridoxine deficiency, and some toxins (25, 384). The syndrome should be differentiated from curly toe paralysis due to riboflavin deficiency.



30.15. Two-day-old turkey poults with splayed legs.

Spraddle Legs

Birds with this condition have one or both legs splayed laterally from the coxofemoral joint (Fig. 30.15). It is usually associated with high humidity during incubation or when newly hatched chicks are placed on slippery floors. The birds have their legs directed laterally and are unable to stand. Affected birds are culled, but this condition may not be manifested until birds are 2–3 weeks of age as the leg deformity becomes obvious.

Miscellaneous Abnormalities of the Spine

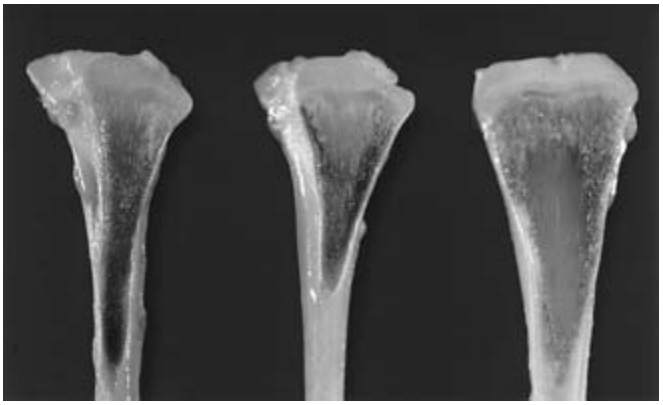
Several other spinal deformities occur sporadically at a low incidence in commercial poultry. These deformities include scoliosis and rumplessness and have been reviewed by Riddell (384).

Crooked Neck

Riddell (393) described a syndrome in turkeys characterized by crooked-neck or wryneck. The major lesions were osteodystrophy of the cervical vertebrae and airsacculitis due to *Mycoplasma meleagridis* (MM). After wryneck was associated with MM infection in the breeders, control measures such as dipping eggs and the implementation of the National Poultry Improvement Plan were put into practice. The condition is rarely seen today. The latest report on this condition involved a back yard turkey flock (52).

Embryonic Cartilage in Ratites

Normal embryonic cartilage can be seen in the long bones including the vertebrae of ratites (ostriches, emus and rheas) between ages 1 day and 8 wk (381, 421, 428). The cartilage which appears like a core and extends from the growth plates from ei-



30.16. Sagittal section of tibiotarsal bone from a young ostrich with embryonic cartilage.

ther end of the long bones in to the metaphysis and diaphysis in birds up to one week of age (Fig. 30.16). As the birds mature, the cores become smaller leaving islands of cartilage in the metaphysis or sometimes in the diaphysis. The femur becomes pneumatic between 6–8 wk of age whereas the tibiotarsus and tarsometatarsus will have normal marrow and trabecular bone (421, 428). Diagnosticians may confuse the cartilaginous cores in the ratites for tibial dyschondroplasia (TD), but it is not TD. TD is a pathologic lesion, whereas cartilaginous cores are normal embryonic remnants of the cartilage that gets resorbed completely by 6–8 wk of age. Microscopically, TD will appear as a sheet of cartilage without interruption by blood vessels, but the cartilage in ratites will have blood vessels scattered through out (421).

Diseases of Muscles and Tendons

Deep Pectoral Myopathy

Deep pectoral myopathy has also been called green muscle disease. Ischemia following exercise in heavily muscled meat-type turkeys and chickens causes the condition. Condemnation of affected muscles has resulted in economic loss in breeder turkeys. The condition was first described in Oregon in breeder turkey hens older than 10 mo of age, with up to 9% of some flocks being affected (97). Several strains of bronze, as well as large, medium, and small white turkeys were affected. Both sexes have the defect (179). The lesion has been recognized in turkeys elsewhere in North America (165) and in the United Kingdom (214). The lesion has also been described in meat-type breeding chickens (181, 213) and in 7-wk-old broiler chickens (383).

Clinical Signs and Pathology

The lesion does not affect the general health of birds and is generally only found at processing. The lesion can be unilateral or bilateral. Chronic lesions result in dimpling or flattening of the breast muscles. These lesions can be detected by palpation (178). Comprehensive descriptions of the pathology of the lesion in turkeys have been provided by Siller and Wight (436), and in broiler breeder chickens by Wight and Siller (497). Lesions in both types of birds are similar. In early lesions, the whole deep pectoral muscle is swollen, pale, and edematous with necrosis in the middle third to three-fifths of the muscle. The overlying fascia is often opaque with edema between the deep and superficial muscles. In older lesions, the edema disappears and the necrotic muscle becomes more prominent and drier with greenish areas. In chronic lesions, the necrotic muscle has shrunk and is uniformly green, dry, and friable and enclosed by a fibrous capsule. It may shrink to a fibrous scar. The muscle posterior to the necrotic muscle becomes atrophied, pale, and sometimes fibrosed. The sternum adjacent to the necrotic muscle is roughened and irregular.

When examined microscopically, the fibers in the green necrotic muscle are swollen and uniformly eosinophilic with discoid necrosis. Nuclei are absent or faint. Blood vessels within the necrotic tissue often contain only nuclei of lysed red blood cells. Surrounding the necrotic tissue, there is an inflammatory reaction with heterophils, macrophages, and giant cells, and in chronic cases, a fibrous capsule. Viable, degenerate, and regenerating muscle fibers are often enveloped by the capsule. Brown pigment and cyst-like structures containing yellow material are also found within the capsule. In the muscle posterior to the necrotic tissue, fibers may be atrophied and replaced by fat, and in some instances, fibrosis is present. Vascular lesions consisting of thromboses, intimal proliferation, and aneurysm formation are found in and around the necrotic tissue. Ultrastructural studies on affected muscles have been conducted (214, 497).

Pathogenesis and Etiology

In a series of experiments Wight (498), Siller (435, 437), and Martindale (274) demonstrated that deep pectoral myopathy is the result of ischemia secondary to the swelling in a tight fascia of a vigorously exercised muscle. In prior studies, surgical occlusion of arteries to the pectoral muscles in both turkeys and chickens resulted in infarcts similar in appearance to the lesions of deep pectoral myopathy (337, 438). In subsequent studies, temporary occlusion of the subclavian artery combined with electrically induced contractions of the deep pectoral muscle induced necrosis of the muscle in both lightweight and broiler strains of chicken. Similar electrically induced contractions alone produced necrosis of the muscle in the broiler strains, but not in the lightweight chickens (498). Subsequently, it was demonstrated that the necrosis could be produced by voluntary wing movements (437). Surgical incision of the fascia around the deep pectoral muscle prior to exercise, however, would prevent development of the lesion (435). Angiography demonstrated a complete ischemia in the deep pectoral muscle associated with an increase in subfascial pressure following electrical stimulation of the muscle. After 24 hr, the ischemia only persisted in the middle of the muscle (274).

It is possible that the high incidence of deep pectoral myopathy in turkey breeder hens is, in part, the result of the extensive handling these birds receive during artificial insemination. Modification of handling procedures may reduce the incidence (495). Some evidence has been produced for a hereditary predisposition (178). This predisposition may be related to inadequate

vasculature in muscles of meat-type birds (498). No specific nutritional factors are known to influence the condition (165, 180), but food restriction may reduce the incidence (497).

Rupture of the Gastrocnemius Tendon

For many years, lameness due to rupture of the gastrocnemius tendon has been recognized commonly in meat-type chickens and rarely in turkeys. It can cause considerable economic loss in broiler breeder flocks and in broiler chickens raised to roaster weights. The early literature on the condition was reviewed by Peckham (354).

Clinical Signs and Pathology

Up to 20% of a flock may be affected. Most outbreaks have been in broiler breeder chickens older than 12 wk of age, but the condition has been recognized in broiler chickens as early as 7 wk of age. The rupture can be unilateral or bilateral. Onset of lameness is acute. Birds with bilateral rupture have a characteristic posture in which the bird sits on its hocks with its toes flexed (Fig. 30.17). In affected birds, a swelling can be palpated on the posterior surface of the leg just above the hock. With acute lesions, hemorrhage can be seen through the skin. With older lesions, there is green discoloration. With chronic lesions, no discoloration may be apparent, but a very firm mass of abnormal subcutaneous tissue can be palpated. Dissection of acute lesions reveals a blood-filled swelling under the skin on the posterior surface of the leg. Within the hematoma, the free end of the ruptured tendon can be found. The rupture generally occurs as an irregular transverse break just above the hock joint. In older and chronic lesions, the blood is partially or completely reabsorbed and fibrous tissue encloses the end of the ruptured tendon and surrounding tissue. Microscopic lesions are variable. In many acute lesions, there is hemorrhage only. In older lesions, there is fibrous tissue surrounding resolving hematomas and the ruptured tendon. Synovial hyperplasia and infiltration of heterophils and macrophages vary from very little to massive. The infiltration of inflammatory cells occurs within the tendon and in the synovial membranes and cavities, and may be associated with masses of heterophil debris and some bacterial colonies.

Pathogenesis and Etiology

Duff and Randall (119) reviewed the literature on the causes of rupture of the gastrocnemius tendon. They concluded that tenosynovitis, in particular that due to reoviruses, may be implicated in some cases. In other cases, the rupture appears to be spontaneous. In such cases, a frequent concurrent finding is rupture of other pelvic limb tendons or ligaments. In cases associated with tenosynovitis, there was a marked inflammatory response, while in spontaneous rupture there was a minimal inflammatory response.

The tensile strength of the flexor digitus perforatus and perforans tendon to the third digit is less in meat-type chickens than in egg-type chickens. It has been suggested that this could predispose meat-type birds to tenosynovitis (464). This could also predispose to spontaneous rupture of tendons. Tissue of the gastrocnemius tendon in meat-type birds has a less organized appearance than that in egg-type birds (465). In addition, many meat-type birds have a hypovascular area in the gastrocnemius tendon just above the hock joint. This hypovascular area is associated with thickened chondrocyte plaques, chondrocyte death, and excessive lipid accumulation in the tendon. These changes may predispose to noninfectious tendon rupture (116). Little research has been conducted on the effect of nutrition on tendon strength. In one study, administration of glycine, vitamin C or E, or copper had no effect on tensile strength of tendons (466). In another study, restricted feeding had no effect on tensile strength of tendons, but the ratio of tensile strength to body weight was less in chickens fed *ad libitum* than in those fed a restricted amount of feed (396). A recently study demonstrated that prolonged sitting by the broilers does not predispose the tendons to ischemia and subsequent necrosis (82).



30.17. Roaster chicken with bilateral rupture of the gastrocnemius tendon. Hock-sitting posture with toes directed ventrally is characteristic. (Craig Riddell)

Diseases of the Circulatory System

Pulmonary Hypertension Syndrome in Broiler Chickens

Pulmonary hypertension syndrome (PHS), also known as ascites syndrome, occurs worldwide in growing broiler chickens and is a significant cause of mortality in many flocks. A low prevalence of the syndrome has been found in most broiler flocks at processing (390). The average incidence of ascites in broiler flocks is 4.7% (309). Interactions between the environmental and genetic factors play a significant role in developing this disease (93). PHS was first reported in flocks of broiler chickens reared at high altitudes in Bolivia (171). It has since been described worldwide in flocks reared at high (51, 79, 195, 269) and low altitudes (136, 234, 390, 449).

Clinical Signs and Pathology

Affected birds usually are smaller than normal and listless with ruffled feathers and pale shrunken comb. Severely affected birds

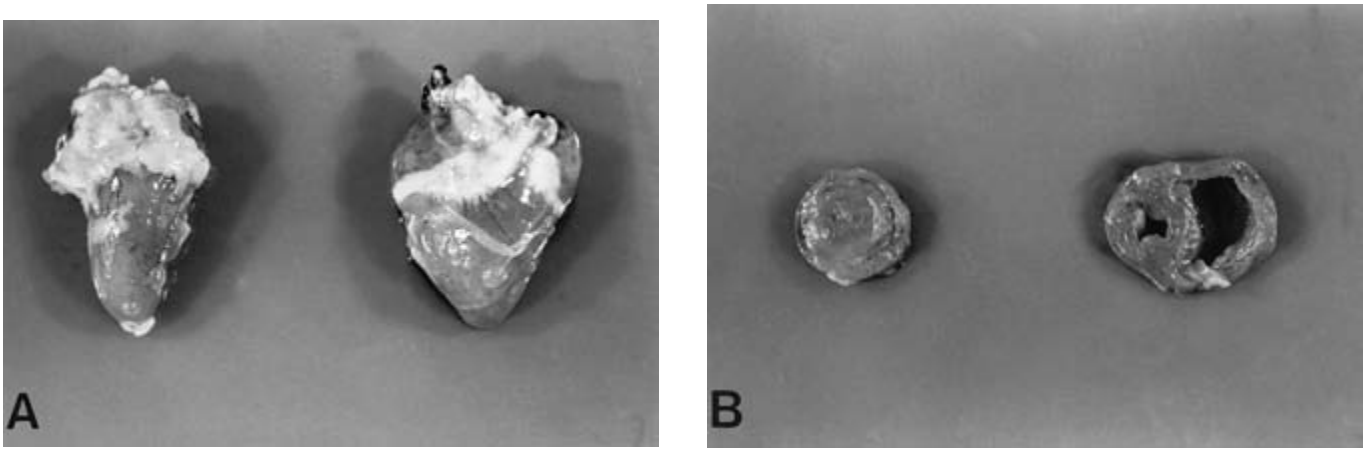


30.18. Broiler chicken with abdomen distended with fluid secondary to right ventricular heart failure. (Craig Riddell)

have abdominal distension (Fig. 30.18), may be reluctant to move, and are dyspneic and cyanotic (287). Some birds may die suddenly before ascites develops (222, 234). Accumulation of straw-colored ascitic fluid with or without fibrin clots explains the abdominal distension (171, 287, 499). Electrocardiogram of affected chickens shows an increase voltage of ventricular depolarization complex, consistent with dilation and hypertrophy of the right ventricle (325, 483). These changes in the echocardiogram are preceded by modifications in the left ventricle consistent with enlargement, progressive left failure and compensatory right ventricular hypertrophy in birds raised at low altitudes (328, 333).

Gross lesions include ascites, right-side cardiac enlargement, often left-side ventricular dilation, and variable liver changes. The cardiac enlargement includes dilation of the right atrium, sinus venosus, and vena cava as well as the right ventricle (Fig. 30.19) and hypertrophy of both the right ventricle and right muscular atrioventricular valve. The ratio of the weight of the right ventricle to the weight of the total ventricles is greatly increased in affected birds (195). Nodular thickening of the atrioventricular valves is characteristic of hearts from ascitic birds (171, 332). Endocardiosis is more common in the left atrioventricular valve than in the right (332). Hydropericardium may be present. The livers in affected birds vary from congested or mottled to shrunken with a grayish capsule and irregular surface. Lungs are congested and edematous (171, 287, 499). Blood from affected birds has increased packed-cell volume, hemoglobin, and red and white blood cell counts. Heterophils and monocytes are increased at the expense of lymphocytes (287). A condition with similar pathologic changes in heart and liver has been reported in meat-type ducklings (221)

Microscopic lesions have been described in the heart, liver, lung, and kidney (171, 287, 309, 499). The myocardial fibers are mildly disorganized, with occasional myocardial degeneration and calcification, edema and some proliferation of loose connective tissue between fibers, focal hemorrhages, and infiltrations of heterophils. The liver has hepatocytic necrosis, dilation of hepatic sinusoids and often fibrosis of the capsule. Foci of lymphocytes and heterophils in the liver are common. The lungs are often hyperemic with visible evidence of hemorrhage, edema, and hypertrophy of smooth muscle around the parabronchi, and collapse of the atria and air capillaries. Increased numbers of cartilaginous and osseous nodules have been found in the lungs of birds affected with ascites (130, 279, 309, 340). However, there was little correlation between the numbers of nodules in lungs and birds that developed PHS (340). The authors hypothesized that hypoxia alone has little influence on the development of pul-



30.19. Right ventricular heart failure in a broiler chicken. A. Enlarged heart (*right*) compared with a normal heart (*left*). B. Transverse section through the enlarged heart showing dilation and hypertrophy of the right ventricle compared with a transverse section through the normal heart. (Craig Riddell)

monary nodules and that the nodules probably are secondary to poor ventilation, increased levels of ammonia and/or dust. Kidneys may have congested glomeruli with thickened basement membranes and scattered foci of lymphocytes (287).

Ultrastructural changes in PHS include myofibril disorganization, mitochondrial abnormalities and hyperplasia in the heart, thickening of alveolar and capillary walls in the lung, and thickened basement membranes and tubular degeneration in the kidney (282, 283, 288). Abnormal calcium deposits have been demonstrated in the mitochondria of cardiac myocytes of birds with PHS (285). Increased serum troponin T found in live birds with PHS was another indicator of myocardial damage associated with PHS (286). An additional ultrastructural observation in some birds with PHS was the presence of viral particles between muscle fibers in the heart (288). These virus particles were identified as retroviruses (350).

Pathogenesis and Etiology

Despite the intensive investigation of the syndrome for many years, the primary cause of ascites is unclear. Pulmonary hypertension causing right ventricular hypertrophy and failure has been considered the main cause of ascites (222). In recent years, it is becoming apparent that the pathogenesis of this syndrome is multifactorial with the interaction of genetic and environmental factors being the most likely causes (93, 231). Because there is a complex interrelationship between the cardiovascular, respiratory, circulatory and other systems in the body, some of the observed changes in ascites syndrome may represent secondary compensatory responses. It is also possible that multiple genetic factors involved in the development and regulation of these systems are key factors that play a role in this complex metabolic disease (369).

Wideman and French (480) showed that animals that survived an ascites challenge produce offspring with reduced ascites syndrome. Body weight has been shown to have a negative genetic correlation at cold temperatures (92, 346). On the other hand, this syndrome had a weak, but positive correlation to body weight when measured under normal conditions. Lubritz *et al.* (271) demonstrated correlations between clinical ascites and coronary arterial pressure index. Birds with a better feed efficiency appear to be slightly more susceptible to ascites (345). Since genetic factors play an important role in susceptibility of birds to ascites syndrome, it offers an opportunity for selection against this syndrome. Alternative selections systems that look into parameters other than just body weight should be considered to reduce PHS. Right ventricular to total ventricular weight of the heart and hematocrit value measured under normal and cold conditions may help to achieve high gain for body weight while keeping PHS level constant (344, 512). Although it is tempting to simply select-out this syndrome without further studies, it is important to understand the molecular mechanisms and genes underlying PHS since these might help improve our insight into the factors that play a role in cardiovascular functioning. Cisar *et al.* (61) found that the concentration of two mitochondrial matrix proteins involved in the aerobic metabolism were elevated in ascites-resistant line broilers with ascites.

Experimentally the two major factors that increase the incidence of PHS are hypoxia and increased metabolic rate (231). In the field the most important environmental factors causing the development of PHS in broilers are high altitudes and cold temperatures (344). Tissue hypoxia may occur at high altitudes, when the oxygen binding affinity with hemoglobin is decreased (79); PHS has been reproduced in hypobaric chambers (226, 341, 342, 503). Hypoxemia results in increased cardiac output, polycythemia, increased hemoglobin, and an increased hematocrit. These changes in the blood produce an increased blood viscosity and larger and more rigid red blood cells, which may have difficulty passing through the capillary bed of the lung, contributing to pulmonary hypertension (284, 300). It has been hypothesized that the modern broiler chicken is susceptible to PHS because its rapid growth causes a higher demand for oxygen (232). In addition, the modern broiler chicken may be more susceptible to hypoxemia because its small lung relative to body size (187, 230, 468), thicker blood-gas barrier (468), and larger and less deformable red blood cells (280, 300). There has been little evidence that reduced environmental oxygen causes PHS at low altitudes (235, 432). Hypoxic conditions induce tachycardia to increase blood oxygenation. While chickens with ascites raised at high altitudes have tachycardia, fast growing chickens with ascites in low altitudes are bradycardic (331), suggesting that different factors trigger PHS.

Several studies have demonstrated that low oxygen levels during incubation influences the occurrence of ascites later in life. Hypoxia during incubation caused lung congestion that was still present at 5 wk (289). Buys et al (50) observed that embryos exposed to high carbon dioxide concentrations during the third week of incubation had lower incidence of ascites during the growth period than those incubated under normal concentrations. On the same lines, Hassanzadeh et al (186) found that birds incubated at high altitude (low oxygen) showed less right ventricular hypertrophy and ascites mortality than those incubated at low altitude. In both studies, authors observed that chickens exposed to less oxygen during incubation hatched earlier than those exposed to normal amounts, and the embryos might have experienced hypoxia for a shorter period of time. They also had higher plasma triiodothyronine and thyroxine (50, 186). Furthermore, embryos incubated at high altitude had higher plasma corticosteroid and lactic acid levels (186) and reduced binding capacity of myocardial beta-adrenergic receptors (185), suggesting an adaptation of the heart to hypoxia.

Abnormal metabolic rate has a direct effect on the incidence of PHS. Low temperatures are also known to induce ascites (10, 344). Chickens susceptible to ascites are believed to be hypothyroid with a limited ability to produce T_4 hormone and a low capacity for oxygen consumption (412). Because of the low concentration of thyroid hormones in the plasma, chickens susceptible to PHS may be unable to meet the increased demand for oxygen at cold temperature (212), resulting in hypoxemia. The addition of 3,3-,5-triiodothyronine to the diet of broiler chickens increased the incidence of right ventricular hypertrophy and the cumulative mortality linked to ascites (94), possibly secondary to an increase in oxygen demand.

The incidence of PHS can be decreased by reducing growth rate (430). Apparently, broilers with restricted feed intakes do not exhibit the bradycardia observed in broilers fed ad libitum (331). The increased cardiac output in the feed-restricted animal may prevent the hypoxia. On the down side, feed restriction reduced breast muscle growth (1). The incidence of PHS is lower in broiler chickens fed mash diets when compared with broiler chickens fed pelleted diets (87, 431), without reducing body weight, weight gain, feed consumption or feed conversion rate (32). Feed restriction reduced the incidence of PHS in birds exposed to low temperature by reducing lipid peroxidation, promoting the activity of enzymatic antioxidant and inhibiting pulmonary vascular remodeling (347).

Accumulation of reactive oxygen may damage the cell membranes of different systems in the body. A direct correlation between low concentration of antioxidant in the lung and enlarged right ventricular weight has been found in broiler chickens (33). Lower levels of antioxidants also have been reported in the lung and liver of broiler chickens affected with PHS (131). This suggests a deficiency in the control of oxidative stress. Dietary supplementation with coenzyme Q_{10} reduced PHS in broilers (149), probably by reducing free radicals.

Several drugs have been used experimentally to reduce ascites. Supplemental L-arginine reduced the incidence of PHS mortality in experimental broilers. This was explained on the basis that L-arginine might be required as a substrate for the production of nitric oxide, a powerful endogenous pulmonary vasodilator (485). On the other hand, nitric oxide may induce pulmonary hypertension by releasing reactive oxygen that may destroy cellular membranes in the vascular and pulmonary system (7, 69). Pulmonary artery hypertension was prevented at low temperatures by blocking the endothelin-1 receptor with BQ123 (510). Angiotensin II may trigger right ventricular hypertrophy even in the absence of pulmonary hypertension (84).

During the development of ascites chickens develop systemic hypotension, which triggers retention of fluid and electrolytes (140). It is not surprising that furosemide, a diuretic that acts as a vasodilator, reduced PHS mortality in experimental broiler chickens, probably by reducing fluid and electrolyte retention, and pulmonary vascular resistance (481).

Systemic acid/base balance also influences the ventilation and perfusion through the lung. Intravenous infusions of 1.2 M HCl led to pulmonary vascular resistance and bradycardia, and could trigger pulmonary hypertension (484). Alternatively, the addition of 1% sodium bicarbonate to a broiler ration to cause alkalosis reduced the incidence of PHS in experimental birds in a hypobaric chamber (343).

Increased resistance to blood flow through the lung can cause pulmonary hypertension, and consequently right ventricular failure and ascites. Pulmonary hypertension in broiler chickens has been induced experimentally by clamping of a single pulmonary artery (482). Clamping of the arteries induces pulmonary hypertension more successfully than occluding the left extrapulmonary bronchus (486); probably because chickens have an effective gas exchange system, but poor blood perfusion through the lung. Inflammatory reaction causes thickening of the gas exchange barrier that may persist even after the causative agent is gone (130). Right ventricular hypertrophy and subsequent ascites are associated with lung damage induced by infectious agents such as *Aspergillus* (223), *Escherichia coli* or infectious bronchitis virus (461); however, pulmonary hypertensive response secondary to exposure to endotoxins or other micro-particles in the lung (56) is linked to genetic factors rather than the source or dosage. Hypervolemia also produces resistance to the blood flow through the lung. Sodium toxicosis resulting in ascites secondary to the increase of blood volume and decrease of erythrocyte deformability (299, 301) should not be mistaken with PHS.

Dilated Cardiomyopathy in Turkeys

Dilated cardiomyopathy (DCM) has commonly been called round heart disease and less commonly the cardiohepatic syndrome. It is desirable that the term *round heart disease* be discontinued, as it is used for a different disease that occurs in chickens (see below); additionally it only describes a gross change but does not indicate the pathology accurately. The early literature on the syndrome has been reviewed by Czarnecki (86) and the reader is referred to this review for more detail and specific citations.

Clinical Signs and Pathology

The highest rate of mortality due to spontaneous DCM occurs in young poults, commonly peaking at 2 wk of age and disappearing at 3 wk of age. The authors have occasionally seen spontaneous dilated cardiomyopathy in turkeys up to 10–12 wk of age. Mortality in flocks averages 0.5% to 3.0% (141), but it has been as high as 22%. Affected young turkeys may die suddenly or may have ruffled feathers, drooping wings, and labored breathing prior to death. On the electrocardiogram, affected turkeys have increased end-diastolic volume, and decrease ejection fraction and systolic blood pressure (148). On postmortem examination, affected young turkeys have greatly enlarged hearts due to dilation of both ventricles. Often, the right ventricle is more dilated. Hydropericardium and ascites may or may not be present. Lungs are generally congested and edematous. Livers may be slightly swollen with rounded edges. In older turkeys from affected flocks the most prominent lesion is enlargement of the heart and hypertrophy of the left ventricle.

Microscopic changes in abnormal hearts are nonspecific and include congestion, degeneration of myofibers, focal infiltration of lymphocytes, and in older turkeys, increased fibroelastic tissue under the endocardium of the left ventricle. Vacuolization of hepatic cells, focal necrosis, bile duct hyperplasia, and intracytoplasmic PAS-positive globules in hepatocytes have been described in the swollen livers.

Pathogenesis and Etiology

Domestic turkeys may be predisposed to the disease due to the abnormal structure of their troponin T (22, 23), an essential protein in the Ca^{2+} regulation of striated muscle during contraction. This discovery is significant as it helps to justify findings from previous research. Abnormal energy metabolism of the heart may cause alterations in the contractility of the heart observed on the electrocardiogram of turkeys with DCM (148). Turkeys with

DCM have a decrease in some enzymes involved in energy supply, such as creatinine kinase, lactate dehydrogenase, Ca^{2+} transport system, ß-receptor-stimulated adenylyl cyclase (148), diminished concentration of ATP (266), and reduced concentration of fatty acids, the main substrate for cardiac metabolism (257). However, what triggers the DCM is still unknown. A genetic influence was demonstrated by breeding trials. Birds with spontaneous cardiomyopathy were selected using electrocardiography, and by mating affected males to affected females, the incidence of the condition was increased in the progeny.

Furazolidone is toxic for turkey poults in concentrations as low as 300 ppm in the feed, and produces a syndrome that is similar to DCM. However, the concentration of polyunsaturated fatty acids in the heart from furazolidone-induced cardiac dilation is markedly increased, while the concentration of these fatty acids in the heart from DCM is markedly reduced (257).

Dilated cardiomyopathy in turkeys has been reproduced with similar environmental and management factors as PHS in chickens. Dilated cardiomyopathy has been related to hypoxic conditions in incubation (81). Clinical observations indicate that the incidence of DCM is increased at high altitude and with cold weather (141). Raising turkeys in a hypobaric chamber at an atmospheric pressure of 592 mm Hg (equivalent altitude 2054 m) resulted in a high incidence of DCM (233). Slowing the growth rate of young poults by dietary manipulation both in a hypobaric chamber at a reduced atmospheric pressure (233) and under commercial conditions (41) reduced the incidence of spontaneous cardiomyopathy. Turkey poults kept at a simulated high altitude on a fast-growth diet developed polycythemia (224), similar to chickens with right ventricular failure and ascites (79). A light program designed to reduce growth rate at an early age also reduced the incidence of spontaneous cardiomyopathy (64).

Sudden Death Syndrome in Broiler Chickens

Sudden death syndrome (SDS) describes a condition in which healthy broiler chickens die suddenly for no discernible cause. The syndrome has been described as sudden death syndrome, heart attack, and flip-over. The latter term has been used because birds dead from the syndrome are commonly found on their backs. The condition was first described as "edema of lungs" in England (189) and subsequently as "died in good condition" in Australia (208). Today, birds dead from SDS are found in most broiler flocks throughout the world. The incidence varies from 0.5 to 4.0% (42, 53, 194, 399, 444). A brief review on SDS was written by Riddell (392).

Clinical Signs and Pathology

Sudden death syndrome has been reported to occur from 1 to 8 wk of age, with the greatest losses occurring from 2 to 3 wk of age in most flocks (42, 399, 444). It occurs more commonly in males than females (330, 335). In a behavioral study, lack of consistent behavioral symptoms did not allow SDS identification prior to death (316). In some broiler flocks, the weekly incidence appears to increase throughout the growing period, suggesting an error in diagnosis or a different syndrome (399). It is possible that birds dying from PHS may have been misdiagnosed as SDS (392).

Affected chickens show no clinical signs or unusual behavior until less than a minute before death. Birds may squawk during a sudden attack characterized by loss of balance, convulsions, and violent flapping (316). Most birds die on their backs with one or both legs extended or raised, but some may die on their sterna or sides (397, 444). Comparison of blood from birds just after death from SDS with blood from killed healthy birds revealed no consistent differences in serum levels of sodium, potassium, chloride, calcium, phosphorus, magnesium, or glucose (397). On the other hand, serum activities of lactate dehydrogenase, glutamic oxaloacetic transaminase (203), and creatinine phosphokinase (368) were increased in SDS chickens compared to healthy chickens.

At necropsy, birds dying from SDS are well fleshed with a full gastrointestinal tract. Livers are enlarged, pale, and friable and, generally, the gallbladder is empty. Kidneys may be pale and the lungs are often congested and edematous (335, 444). The congestion and edema of the lungs may be a postmortem artifact, as it is not found in freshly dead birds (397). The ventricles of the heart are generally contracted and the thyroid, thymus, and spleen may be congested; there may be hemorrhages in the kidney (335).

Microscopic lesions reported are nonspecific. Degeneration and infiltration of lymphoid cells and heterophils have been described in the heart (335). However, the cellular infiltrations have been considered to be normal lymphoid foci and foci of ectopic hemopoiesis (397). Use of an allochrome stain and a hematoxylin-basic fuchsin-picric acid stain did not demonstrate any degenerative changes in hearts of birds dying from SDS (397). In contradiction to these previous studies of the heart in SDS, a more recent study described arteriosclerotic changes and myocardial necrosis mostly in the left ventricle of broiler chickens that had died suddenly without clinical signs (236). The birds studied were 34 to 64 days of age, older than when most SDS mortality occurs and than when birds were examined in one of the prior studies (397). There were also congestion, edema, and lymphoid cell infiltration in the lungs; hemorrhages in the kidneys; and mild bile duct hyperplasia and periportal lymphoid infiltration in the liver. In a study of organ weights, relative liver weights of broilers dead from SDS were significantly greater than the liver weights of control birds, but no significant differences were noted between SDS and control birds in relative weights of lungs, hearts, and intestines (39).

Pathogenesis and Etiology

The pathogenesis of SDS is not understood. Sudden death syndrome was associated with acute cardiac dysrhythmia, consistent with ventricular fibrillation (329). Birds that later died of SDS had a higher heart rate than the rest of the flock (333). Furthermore, a higher percentage of chickens that died with SDS had cardiac arrhythmias (330). It has been suggested that SDS is a metabolic disease and that genetic, nutritional, and environmental factors may affect the incidence (397). Lactic acid or acid-base balance does not appear involved in SDS (209). Sudden death syndrome has been described in most modern broiler-type chickens (42, 399), but the heritability is low (55).

Similar susceptibility to SDS was found between six strains of commercial broiler chickens, including Arbor Acres, Avian Farms, Cobb-500, Hubbard-Peterson, ISA and Ross (156). The incidence of SDS was higher in birds fed crumble-pelleted feed compared with those fed mash feed (366). The authors suggested that this difference in mortality might be associated with changes in the pelleted feed and not with increased growth rate. Incidence of SDS was not affected by growth rate under field conditions (399). Severe feed restriction eliminated SDS in a small experimental trial (40), but restriction of feed intake for 7 days early in the life of broiler chickens did not significantly reduce the incidence of SDS (401). A lighting program in which birds were exposed to a short photoperiod at an early age decreased early growth rate and also decreased the incidence of SDS (63, 394).

Several nutritional factors have been studied with regard to the incidence of SDS. In a field survey, a higher incidence of SDS was noted in flocks fed wheat-based rations compared with flocks fed corn-based rations. This difference was also noted in some experimental trials (26), but no differences were noted in other nutritional trials (199, 302). Protein type and concentration in the diet influence the incidence of SDS. Chickens fed meat meal protein had lower incidence of SDS than those fed soybean meal (26). Also, higher percentage of protein in the finisher diet reduced the incidence of SDS (302). Addition of vitamins did not affect the incidence of SDS (199, 302, 367, 445, 478). Lipid metabolism may affect cardiac sarcoplasmic reticular transport (60). Increased amounts of calcium, phosphorus, and magnesium (220) or potassium (199) in rations had no effect on the incidence of ADS. In contradiction to some of these previous studies, a more recent study indicates that increase in the dietary calcium and phosphorus boost the susceptibility to SDS. A calcium antagonist, verapamil, added to the diet had no effect on the incidence of SDS (158). It has been suggested that thiamine may influence the incidence of SDS (62).

Flock size has been proposed as a factor that might influence the incidence of SDS (399). High stocking density can increase the occurrence of SDS (204). An experimental study showed that light intensity did not affect the incidence of SDS (319), but a field trial suggested that intermittent light may decrease the incidence of SDS (336). Extended dark periods (more than 8 hours) also reduced SDS, but had negative effect on body weight (414). Decrease in SDS may be associated with lower heart rates during dark periods (27). Increased light intensity alternating from side to side within pens, when superimposed on a background of low light intensity, had no effect on the incidence of SDS (317, 318).

Aortic Rupture

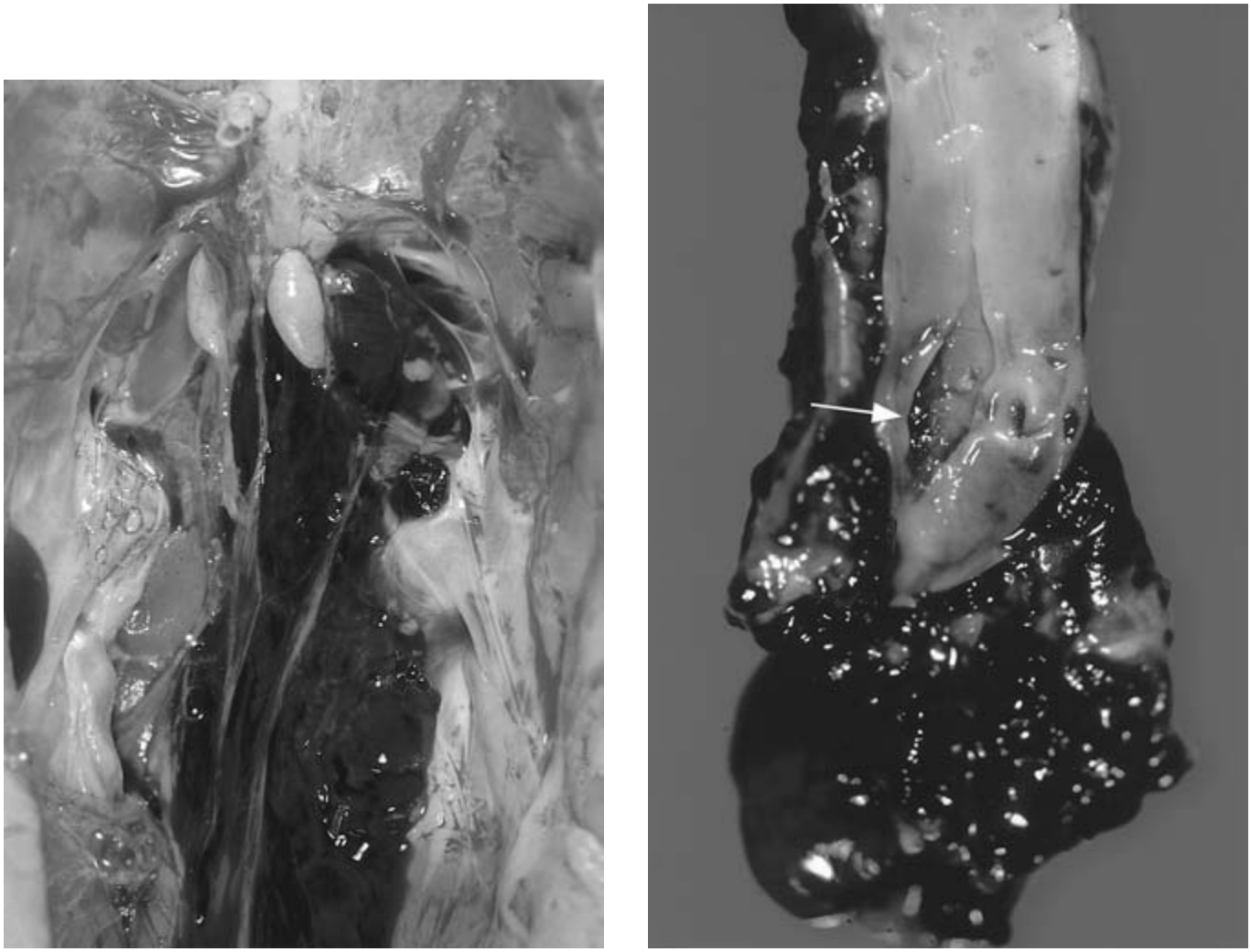
Aortic rupture or dissecting aneurysm is characterized by sudden death in growing turkeys due to internal hemorrhage. The condition has been recognized throughout North America, in Europe, and in Israel. Mortality in the past has been reported to reach 50%, but losses in affected flocks at present usually only reach 1–2%. Aortic rupture also has been described in ostriches and emus (446).

Clinical Signs and Pathology

The condition occurs in turkeys between 7 and 24 wk of age, with a peak mortality between 12 and 16 wk of age. The incidence is primary in male turkeys. Affected birds die suddenly. Gross and microscopic lesions have been described by McSherry *et al.* (295) and Pritchard *et al.* (365). At necropsy, the head, skin, and musculature are anemic. Occasionally, blood will run out of the mouth, or the oral cavity will be bloodstained. Upon internal examination, large clots of blood will be found in the abdominal cavity and beneath the capsule of the kidney. Clotted blood may be present in the pericardial sac, lungs, and leg muscle. Aneurysm and rupture of other arteries, such as the coronary artery, have also been described (425). In the ruptured aorta, a longitudinal slit is present between the external iliac and ischiatic arteries (Fig. 30.20). In one study a linear or semi-linear tear was found in the aorta at the origin of the coeliac artery in majority of the turkeys (429). In this region, the aorta is dilated; the wall is thin and has lost its elasticity. The tunica intima and media may be thrown into deep folds and partially separated from the tunica adventitia. Fibers of the tunica media may show mild to severe degenerative changes and may be infiltrated with heterophils and macrophages. The media may be thickened due to an increase in ground substance and fibroblastic proliferation. Dissolution or disappearance of the elastic laminae of the media occurs at the site of rupture. Degenerative changes and areas of erosion and cellular infiltration may be present in the adventitia. A marked intimal thickening or a large fibrous intimal plaque often occurs in the region of rupture. Sudan II stains reveal lipid accumulations in the affected intima.

Pathogenesis and Etiology

Several reports have emphasized the possible role of intimal plaques in the pathogenesis of aortic rupture in turkeys. It has been suggested that these plaques and the absence of an intramural vasa vasorum around the abdominal aorta result in impaired nutrition to, and degeneration of, the media (313). High blood pressure in young male turkeys may also be a precipitating factor, but paradoxically, the administration of diethylstilbestrol decreased blood pressure and increased the incidence of aortic aneurysm (244, 245). Diets containing high levels of protein and fat may increase the incidence of aortic rupture (365). Low copper levels were found in the livers of turkeys from field outbreaks of aortic rupture (157) and coronary artery rupture (425). Like in turkeys, aortic rupture in ratites has been associated also with copper deficiency (467). Copper is important in collagen synthesis and it has been suggested that copper deficiency may play a role in aortic rupture. Because the incidence of aortic rupture occurs primarily in male turkeys and copper deficiency has not been consistently found in birds with aortic rupture, genetic diseases such as connective tissues disorders of the elastin and/or collagen should also be investigated. Beta-aminopropionitrile, a toxic product that occurs in the sweet pea (*Lathyrus odoratus*), will produce aortic rupture in the turkey but has not been incriminated in the field syndrome (354). Uncontrolled field studies suggest favorable results in treatment of ruptured aorta with reserpine. This has not been confirmed experimentally and such treatment may depress growth rate (354).



30.20. Aortic rupture. Left. Abdominal bleeding, around the kidneys, secondary to ruptured artery in a 16-wk-old meat turkey. Right. Tear of the aorta wall (arrow) at the level of the ischiatic artery.

Sudden Death in Turkeys Associated with Perirenal Hemorrhage

Sudden death in turkeys associated with perirenal hemorrhage (SDPH) has been recognized as a significant cause of mortality in male turkeys between 8 and 14 wk of age in many areas of North America (143, 307, 504). It was first recognized in Israel in 1973 (312). Documented mortality due to SDPH varies from 0.8 to 1.80% (143, 504). Estimates of mortality as high as 6% have been made (307). The syndrome has also been described as hypertensive angiopathy (225, 247).

The dead turkeys are in good condition, with food in their crops and the remainder of the gastrointestinal tract. They have congested and edematous lungs, splenomegaly, congested livers and digestive tracts, and clotted blood surrounding a portion or the whole of the kidneys (142, 254, 307, 504). Perirenal hemorrhage is not a consistent lesion (504). The most significant gross lesion is probably cardiac hypertrophy affecting the left ventricle and intraventricular septum (142, 254). Microscopic lesions include congestion in various organs, with edema in the lungs and hemorrhages in the lungs and kidneys (142, 254). Arterial lesions including internal vacuolation and medial hyperplasia have been found in affected turkeys (142, 225), but have also been recognized in normal control turkeys (307). Hyperplasia of the epithelium and a decrease of colloid in the thyroid glands were noted in the only study in which these glands were examined (254).

It has been proposed that the cause of death in SDPH is acute congestive heart failure secondary to cardiac hypertrophy. The renal hemorrhage may result from severe passive congestion, which may be compounded in part by closure of the renal valve in the renal portal circulation (254). The thyroid hyperplasia may contribute to the hypertrophic cardiomyopathy (254). Hypertension is common in young male turkeys (244) and may explain the cardiac hypertrophy and vascular lesions. It has been postulated that poor exercise tolerance in the modern turkey may result in cardiac arrhythmias and SDPH (38). Male turkeys have greater relative left and total ventricular weights than those of females of

the same age. This might explain the greater susceptibility of male turkeys to SDPH (37). In the opinion of the authors, perirenal hemorrhage in turkeys may be related to aortic rupture. This is based on the observations that many birds that died during an episode of aortic rupture also had perirenal hemorrhages. Careful dissection of the posterior abdominal aorta and its branches in the vicinity of the kidneys revealed microscopic lesions in them. Occasionally small tears or ruptures could be seen grossly in the posterior abdominal aorta and its branches; renal, external iliac and ischiatic arteries.

Fast weight gain, continuous lighting programs, crowding, and hyperactivity have been suggested as factors that may influence the incidence of SDPH (307). Increased room temperature, toe clipping, step up/step down lighting, and dietary reserpine reduced the incidence of SDPH (144). Dietary aspirin had no effect on the incidence of SDPH (36, 144).

Miscellaneous Diseases of the Cardiovascular System

Atherosclerosis

Atherosclerosis is a common disorder of the aorta and other major arteries of the domestic poultry (167, 243) and occasionally pigeons (364). It is more common in males than in females (248). Atherosclerotic changes can be found in birds at any age, but older birds have more severe lesions (242).

The amount of lipid in the lesion is variable. In the most severe cases, thickening of the aorta can be seen grossly. In milder cases, atherosclerosis can be detected grossly with the aid of Sudan IV stain. Microscopically, there are extracellular and intracytoplasmic fatty vacuoles in the smooth muscle of the inner tunica media. The tunica intima is thickened with accumulation of diffuse fatty streaks. In addition, mucopolysaccharides that can be demonstrated with special stains, such as toluidine blue, are abundant in the intima. Abundant fibrous tissue forms a cap over the lesion. Numerous macrophages and occasional mineral deposits are also found in the atherosclerotic plaque.

Atherosclerotic plaques protrude into the aortic lumen, retarding blood flow, and increasing surface tension. It has been suggested that in turkeys these changes may predispose to aortic rupture; however, the relation of atherosclerosis to aortic rupture has not been determined. Hypertension has been associated in atherogenesis (30); but drugs, such as reserpine that lowers arterial blood pressure and the incidence of aorta rupture (354), do not affect the extent of atherosclerosis. Atherosclerosis has been reproduced in chickens with Marek's disease virus and has been used as a model to study human atherosclerosis (see Chapter 15, "Neoplastic Diseases").

Endocardiosis

Endocardiosis is the nodular thickening or fibrosis on the free edges of the atrioventricular valves. It is more commonly seen in the left atrioventricular valve than in the right. Only occasionally is it found in the ventricular endocardium under the atrioventricular valves. It is a common lesion in old birds and a frequent incidental finding during necropsy. In a recent study, endocardiosis was observed in commercial chickens as young as 7 days (332). In the same study, the incidence of endocardiosis was 18% in normal birds, and up to 52% in birds with ascites. The cause of endocardiosis is not known. Severe endocardiosis may cause atrioventricular stenosis and predispose the bird to ventricular dilation (332). Endocardiosis of the right atrioventricular valve is common in chickens with ascites syndrome. Endocardial fibrosis in the left ventricle of chickens has also been described and has been observed by the authors (89).

Rupture of the Right Auricle

Mortality of 3.4% in a flock of 11,500, 10- to 14 day-old broiler chickens due to sudden death attributed to spontaneous rupture of the right auricle has been reported (35). Most of the birds necropsied had hemopericardium and the auricle rupture was present at the junction of the vena cava and the right auricle. The cause of this condition could not be determined.

Round Heart Disease in Chickens

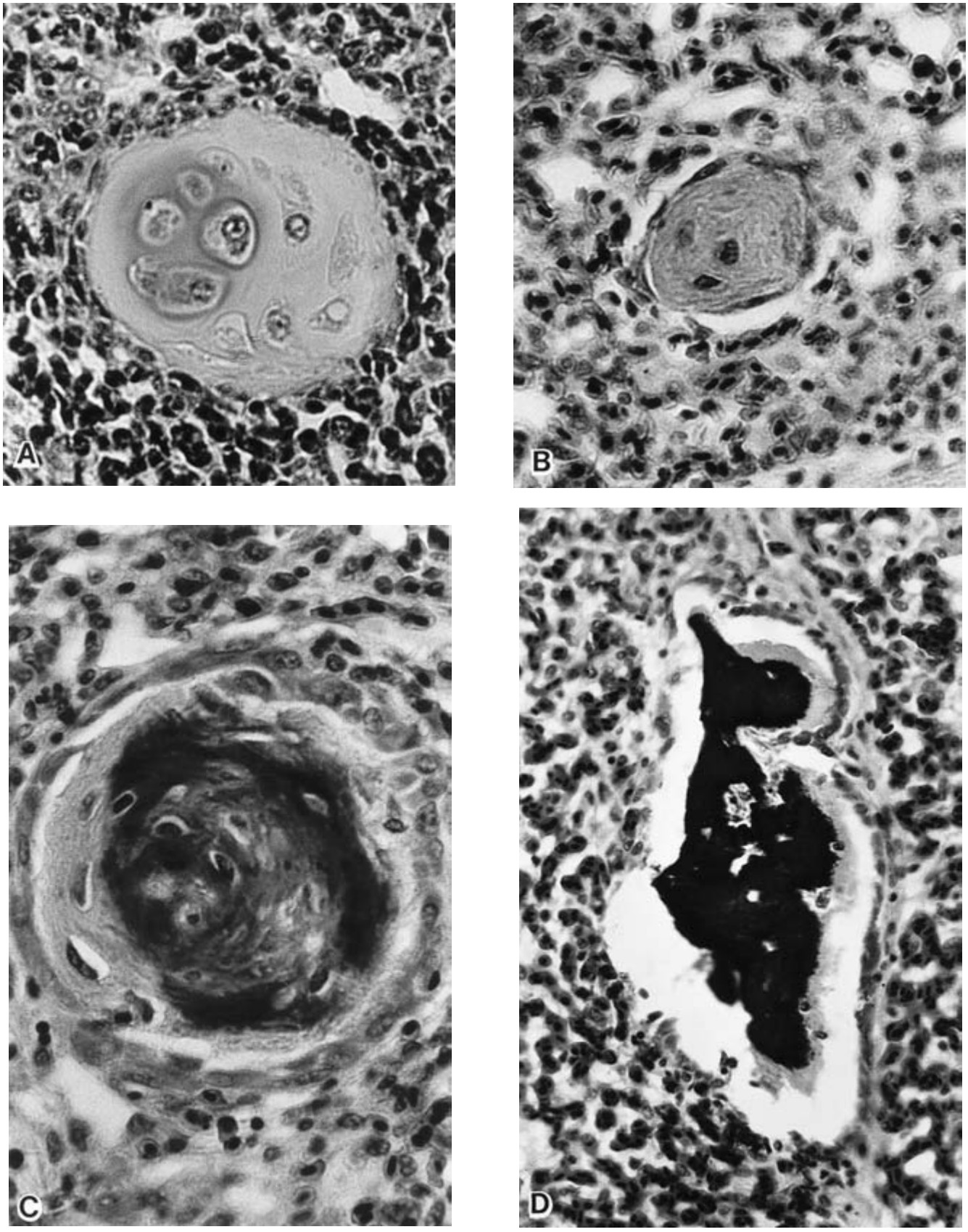
Round heart disease is an acute cardiac failure due to myocardial degeneration in chickens commonly between 4 and 8 mo of age. It used to have a worldwide distribution but has not been reported in commercial poultry flocks for 25 yr. This condition has been reviewed by Riddell (393). Gross lesions consist in enlargement and yellow discoloration of the heart. The apex of the heart is blunt and may be dimpled. Both ventricles are hypertrophied. In some birds, there may be excess gelatinous fluid in the pericardial sac and in a few birds excess fluid is present in the abdominal cavity. Lungs are often edematous and the liver, kidneys, and spleen may be congested. Microscopically there is fatty change in the myocardium. Round heart disease in chickens may be due to a nutritional deficiency or marginal rations.

Diseases of the Respiratory System

Cartilaginous and Osseous Lung Nodules in Broiler Chickens

Cartilaginous and osseous nodules have been reported in the lungs of domestic birds for more than 50 yr, most commonly in the lungs of broiler chickens (227, 279, 407, 494). The nodules are microscopic in size, found in the parenchyma of the lung, and located some distance from large airways and blood vessels. No reaction is visible around hyaline cartilaginous nodules, but the other types of nodules may be surrounded by a thin layer of fibrous cells, heterophils, and macrophages. The nodules appear to change with age from hyaline to fibrous to mineralized cartilaginous types, and finally, to an osseous type (Fig. 30.21). They are more numerous in the left lung than in the right (281), and in males (494).

The cause and significance of these nodules are unknown. It is probable that the nodules may be derived from chondrocytes displaced from nearby bronchi during early development (494). Nodules might be the result of poor ventilation, increased levels of ammonia and/or dust (340). The number of nodules is greater in broiler chickens fed *ad libitum* than in broiler chickens fed a restricted amount of food (407). An increased incidence of nodules has been described in birds suffering from ascites and right heart failure (279, 281, 499).



30.21. Cartilaginous and osseous nodules in microscopic sections from the lungs of broiler chickens. A. Hyaline cartilaginous nodule. H & E, ×528. B. Fibrous cartilaginous nodule. H & E, ×528. C. Mineralized cartilaginous nodule. H & E, ×528. D. Osseous nodule. Van Kossa stain, ×528. (*Avian Diseases*)

Emphysema

Ridell (393) reviewed subcutaneous emphysema or "windpuff." This condition is caused by an injury or defect in the respiratory tract that permits accumulation of air beneath the skin. Subcutaneous emphysema is rarely observed today in commercial poultry, since caponization is rarely or not done at all. Puncturing the skin with a sharp instrument can alleviate this condition. In aquatic or flying birds, some of the pneumatic bones such as the humerus, coracoid, and sternum may fracture, allowing air to accumulate beneath the skin.

Diseases of the Digestive System

Pendulous Crop

Pendulous crop occurs at a low incidence in many chicken and turkey flocks. In severely affected birds, the crop is greatly distended and full of feed, particles of bedding, and fluid, which often has a foul smell (Fig. 30.22). The lining of the crop may be ulcerated. Birds continue to eat, but digestion is impaired and birds become emaciated and die. Carcasses of affected birds are generally condemned at processing. The possibility that diet may influence the incidence of pendulous crop is supported by the experimental production of pendulous crops with rations containing cerelose as a substitute for starch. It was suggested that increased liquid intake in hot weather may be responsible. Additionally hereditary predisposition has been proposed in turkeys. Neither of these factors appears to be important. For further discussion on the possible etiologies of pendulous crop review the 8th edition of this book (354).



30.22. White, broad breasted, meat turkey with pendulous crop.

Impaction

Impaction of the crop, proventriculus, or gizzard has occasionally been reported in poultry, waterfowl, and ratites. The condition is rare in chickens. Gizzard impaction can cause high mortality during the first 3 wk of life in turkey flocks. Proventricular and gizzard impaction are common in ostrich and other ratite chicks. Affected birds are emaciated, with empty intestinal tracts, but affected crops, proventriculi, and/or gizzards are full of a solid mass of interwoven fibrous material. This fibrous mass often extends into the first part of the duodenum, and in some birds, masses of fibrous material are found lower in the intestine. The impaction results from the birds eating litter or fibrous material that the crop, proventriculus, or gizzard is unable to handle. In ratites impaction due to foreign bodies such as rocks, metallic objects, pebbles, etc is common. A recent report of crop impaction due to feather eating has been associated with improper management (305). Prevention is aimed at discouraging the eating of litter or fibrous materials by young poults and ratite chicks.

Dilation of the Proventriculus in Chickens

In 4-wk-old chicks fed a purified diet, dilation of the proventriculus was first reported as proventricular hypertrophy by Newberne *et al.* (314). The abnormality is commonly observed as an incidental finding in broiler chickens. Occasionally, a high incidence in a broiler chicken flock may cause significant carcass contamination when enlarged proventriculi rupture at processing. The enlarged proventriculi have greatly dilated thin walls and are full of feed. The gizzards in affected birds are poorly developed and there is no sharp demarcation between the gizzard and proventriculus (Fig. 30.23). The poor development of the gizzard is generally the result of a finely ground diet lacking in fiber, and the dilation of the proventriculus is secondary (385). This condition must be differentiated from transmissible proventriculitis in chickens the cause of which is not known and from Marek's disease. In Marek's disease, the proventriculus can be dilated occasionally due to infiltration of lymphocytes and plasma cells in the splanchnic nerves and in the subserosal nerves and ganglia of the proventriculus. Reticuloendothelial virus can cause similar lesions in chickens and turkeys.

Intussusception and Volvulus

These conditions are occasionally seen in ratites and sporadically in domestic fowl. Intussusception occurs most frequently in the intestine, but it has been reported also in the proventriculus (417). Volvulus occurs when there is torsion of the intestine around itself or the root of the mesentery. In young birds volvulus of the small intestine may be caused by twisting around the yolk sac (446). Intussusception and volvulus have been reported in chickens secondary to enteritis or abnormal peristalsis caused by nematode or coccidial infection (354); the authors have seen intestinal torsion associated with pedunculated neoplastic stalks. The clinical signs are anorexia and progressive weight loss, and death occurs over a few days. The affected and distal portion of the intestine are severely congested due to circulatory compromise and the intestinal epithelium becomes necrotic rapidly. If an early diagnosis is made, resection of the affected intestine can be performed in a valuable bird.

Cloacal Prolapse

Cloacal prolapse may involve the intestines, reproductive tract (oviduct or phallus), and ureter(s). The prolapsed tissue has a



30.23. Gizzards and dilated proventriculi from broiler chickens fed only a commercial broiler starter (*left*) compared with those from broiler chickens fed a broiler starter containing oat hulls (*right*). The former gizzards are small and the proventriculis enlarged. (*Avian Diseases*)

smooth surface and is shiny and congested. Cloacal prolapse may be associated with diarrhea, impaction, or nutritional imbalances. It occurs commonly in young ostriches and it has been associated with *Cryptosporidium* sp. infection (19). In laying hens, cloacal prolapse may result from egg laying. In poultry, picking of the prolapsed tissue may result in cloacal rupture and evisceration.

Diseases of the Liver

Fatty Liver-Hemorrhagic Syndrome

Fatty liver-hemorrhagic syndrome (FLHS) occurs sporadically in commercial layers (442). It has been recognized in many countries of the world. The syndrome occurs primarily in birds kept in cages, but has also been recognized as a less significant problem in birds kept on litter. It is associated with birds fed high-energy diets, and most often seen during the summer.

Clinical Signs and Pathology

The first sign of the syndrome is an increase in mortality of the flock (49), with birds in full production being found dead with pale heads. Mortality usually does not reach 5%. There is often a sudden drop in egg production. Hens may be overweight, with large pale combs and wattles. Dead birds have large blood clots in the abdomen, arising from the liver and often partially enveloping the liver (Fig. 30.24). The liver is generally enlarged, pale, and friable; it may have smaller hematomas within the parenchyma. These hematomas may be recent and dark red, or older and green to brown. Similar hematomas may be seen in clinically healthy birds in the same flock if such are examined during or after an outbreak. Large amounts of fat are present in the abdominal cavity and around the viscera. Most of the birds have active ovaries and often with an egg in the oviduct.

Microscopic examination of the liver shows hepatocytes distended with fat vacuoles, hemorrhages of various sizes and organizing hematomas, and often small irregular masses of uniform eosinophilic material, likely derivative of plasma protein (496). The fat content of livers generally exceeds 40% dry weight and may reach 70%. The content of phospholipids in the liver are decreased, while the oleic and palmitoleic acids are increased (207). These fatty acids probably are synthesized, since neither is normally found in the diet.



30.24. Fatty liver-hemorrhagic syndrome. A large blood clot is molded over the left lobe of the liver. Note the excess abdominal fat.

Biochemical evaluation of the plasma reflects cellular changes that occur in different organs. Birds from a FLHS-susceptible strain or commercial birds fed diets that induce FLHS had increased concentration of aspartate aminotransferase and other plasma enzymes (96, 511). This is not surprising since the selected enzymes are common indicators of hepatic disease. Laying birds normally have higher levels of estradiol, calcium, and phosphorus in the plasma than inactive birds. However, birds affected with FLHS have even higher concentrations of this hormone (169) and minerals (175) in the plasma than unaffected ones. No changes have been found in concentrations of progesterone (169), major plasma proteins or glucose.

Pathogenesis and Etiology

The pathogenesis and cause of the hemorrhage has not been defined. Factors involved in FLHS were discussed by Squires and Leeson (442) and Hansen and Walzem (172). Excessive energy in the diet induces FLHS regardless of the source. Excessive consumption of high-energy diets in birds whose exercise is restricted in cages is considered to result in a positive energy balance and excessive fat deposition. Excessive fat may disrupt architecture of the liver and result in weakening of the reticular framework and blood vessels in the liver. A pathogenic relationship between hepatic steatosis and hemorrhage has been suggested (352). Lysis of the reticulin framework of the liver has been reported in FLHS. A strong association of reticulolysis with severity of liver hemorrhage has been described in experimental birds. Rupture of intrahepatic portal veins associated with degenerative changes in the veins was described in the same birds (277). Focal necrosis of hepatocytes leading to vascular injury has been described as another mechanism to explain the hemorrhage (202, 509). It has been postulated that excessive lipid peroxidation of unsaturated fatty acids in the liver may overwhelm cell repair mechanisms and result in tissue damage (442).

Because energy balance is a factor in FLHS, many studies have investigated the influence of the diet composition on this syndrome. The incidence of this syndrome increases as the total energy in the diet increases, regardless of the source. However, when isocaloric diets were compared, a diet that provided the energy in fat rather than in carbohydrates reduced the occurrence of this syndrome (170). It was hypothesized that diets richer in fat might have reduced the hepatic metabolism by reducing *de novo* fatty acid synthesized in the liver. Furthermore, size of the liver, rather than lipid content in the liver or high-fat diets, was directly related to induction of FLHS (413). Some by-product ingredients such as dried brewer's grains, dried brewer's yeast and torula yeast, fishmeal, and fermentation by-products reduce the incidence and severity of FLHS (443). Although not indicated in the study, the reduction of FLHS could be due to the high selenium content of these nutrients. High levels of selenium, vitamin E, and other antioxidants reduce the peroxidation of lipids and may reduce the incidence of FLHS (442). Quail fed diets designed to induce hepatic steatosis and limit biologic oxidant defenses developed a syndrome similar to FLHS in chickens. Liver hemorrhage in these quail was reduced by adding vitamin E to the diet, but not by adding glutathione (441). In an attempt to test this postulate in chickens, the diet of a strain of chickens susceptible to FLHS was supplemented with ascorbic acid, tocopherol or L-cysteine. None of these compounds, all of which have a role against oxidation, prevented FLHS (95).

In several reports, mortality in laying hens due to liver hemorrhage has been associated with the use of rapeseed meal in the diet (353). As this may occur without the development of fatty livers, it may be a separate syndrome (442). In addition, rapeseed meal has been shown experimentally to increase the extent and severity of liver hemorrhage, but in these experiments, liver hemorrhage also occurred in birds not fed rapeseed meal (277, 353). The possibility of toxins causing FLHS should not be ignored. Aflatoxin has been considered as a possible cause but produces different liver lesions.

The fat content in the liver normally rises with the initiation of egg production and is influenced by estrogen. Injection of immature chickens with estradiol has been shown to result in hepatic steatosis and hemorrhage (351). Similar injection of laying hens caused liver enlargement, death from liver hemorrhage, and neurologic disorders (443). The observation of greatly elevated plasma estrogen, calcium, and cholesterol in chickens from flocks with FLHS suggests that the syndrome may be due to a hormone imbalance (169, 177, 297). In one experiment, treatment with synthetic estrogens were more damaging at 34°C than at 21°C (3), which agrees well with the field observations that FLHS is more frequent during the hot weather. Additionally, birds exposed to high temperatures are more likely to be in a positive energy balance.

Hemorrhagic fatty liver syndrome in chickens must be differentiated from hemorrhage due to rupture of the liver associated with hepatitis E virus in chickens (see "Hepatitis E Infections" in Chapter 14). Livers are generally not fatty in ruptured livers of chickens associated with hepatitis E virus.

Hepatic Lipidosis of Turkeys

Hepatic lipidosis of turkeys is a disease of uncertain, but probable nutritional, etiology. It has been reported only in turkey breeder hens between 12 and 24 wk of age. The disease has also been referred to as acute hepatic necrosis. Sporadic cases of the disease have been seen in Canada and the United States for the past several years, but only two cases have apparently been described in the literature (147).

Clinical Signs and Pathology

The disease is characterized by an abrupt increase in mortality that may approach 5% during a 1- to 2-wk period. For a brief period prior to death, affected hens may become inactive and show dyspnea and cyanosis; most often, birds are just found dead. At necropsy, carcasses are in good condition with obvious fat deposits especially in the body cavity. The liver is enlarged and has a variable number of sharply contrasting pale yellow and dark red areas (Fig. 30.25). Other findings include petechial or ecchymotic hemorrhages in fat and on organs surfaces, pulmonary congestion and edema, and blood that fails to clot.

Microscopically, livers are characterized by multifocal coalescent central lobular hepatic necrosis and lipid vacuoles in the cytoplasm of hepatocytes around the portal areas. Necrosis and



30.25. Hepatic lipidosis in a 17-wk-old turkey hen. There is mottling and swelling of the liver. There are scattered, well-demarcated, large, pale foci on the liver. Bar = 1cm. (Richard P. Chin)

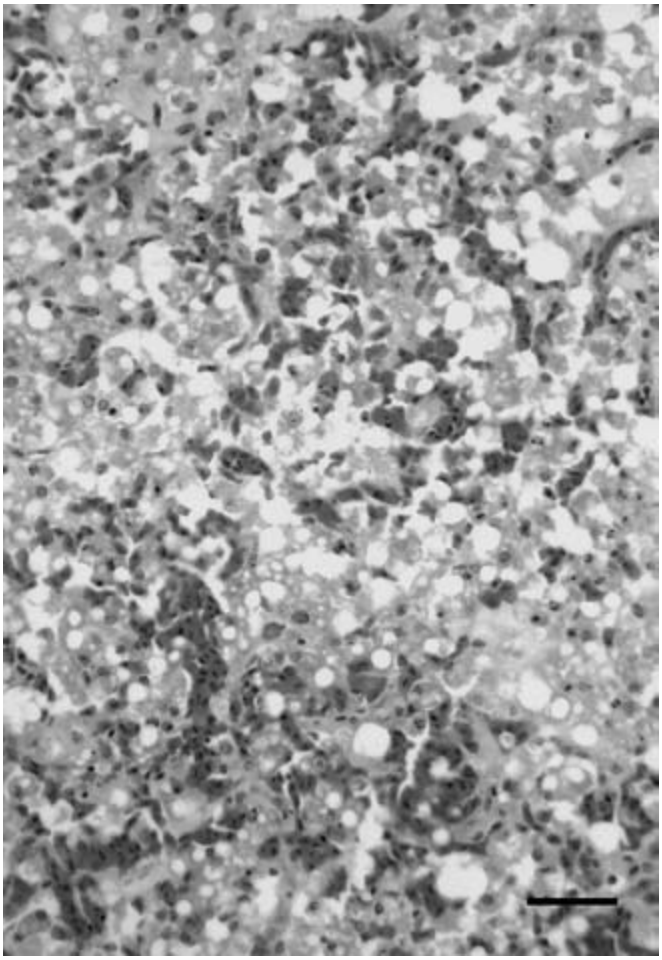
hemorrhage associated with vascular damage are also present. Large confluent areas of fatty degeneration represent pale areas seen grossly, while areas of necrosis and hemorrhage are responsible for dark red areas (147). Occasional nuclei of degenerating hepatocytes contain prominent eosinophilic nucleoli, which could be mistaken for viral inclusion bodies (Fig. 30.26).

Pathogenesis and Etiology

The cause of hepatic lipidosis is unknown, although nutritional, environmental, and management factors are considered to be involved. Affected flocks are typically on low-protein diets to control growth and development. These diets also may be low in lipotropic factors, especially methionine and cysteine, while still having high energy levels. These amino acids are required for production of apolipoproteins. High peroxide values also have been associated with the disease. High environmental temperatures and/or changes in lighting programs cause the birds to alter eating habits, leading to hepatic deposition of lipid and, eventually, to liver failure. Terminally, lipid peroxidation is considered to contribute to vascular damage, which results in lung edema and liver necrosis and hemorrhage (147).

Picornavirus-like particles suggestive of avian encephalomyelitis (AE) virus and low numbers of *Escherichia coli* have been identified in the affected livers (57). These are believed to be incidental because turkey breeder hens in this age range are typically vaccinated for AE using live viruses, and it would not be unusual to recover bacteria from livers of seriously sick or dead birds.

Administration of vitamin E to affected flocks (25 IU/hen) via water for 7 days reduced mortality (147). Hepatic lipidosis is prevented by having adequate methionine (0.2%) and methionine plus cysteine (0.4%) in the ration (311). Prevention of the disease



30.26. Photomicrograph of liver from a 17-wk-old turkey hen with hepatic lipidosis. Hepatocytes are vacuolated and with fatty degeneration. There is severe hemorrhaging and biliary hyperplasia. H & E, bar = 20 μm. (Richard P. Chin)

was achieved by supplementing the standard feed with 1 kg 60% choline, 1 kg methionine, and 20 g vitamin B_{12} per ton (147). In some areas, apparent successful prevention of the disease has followed use of a protocol in which the initial AE vaccine is given via water followed by booster inoculation in the wing-web instead of giving two wing-web inoculations.

Diseases of the Urinary System

An excellent review of renal pathology of the fowl was written by Siller (434). This discussion covers only metabolic conditions of major importance commonly seen in commercial poultry. It does not cover descriptions of miscellaneous conditions such as congenital malformations and baby chick nephropathy. Urate deposition and urolithiasis together are responsible for significant losses in poultry. An increased substrate load on the kidney that leads to dysfunction of this organ with precipitation of insoluble products within the kidney itself or other organs can cause urate deposition or urolithiasis.

Urate Deposition ("Gout")

Uric acid is produced in the liver and is the end product of nitrogen metabolism in birds. Consequently, birds can develop urate deposits secondary to an abnormal accumulation of urates. Urate deposition should not be considered as a disease entity, but as a clinical sign of severe renal dysfunction that causes hyperuricemia. Clinicians and diagnosticians alike tend to use the historical terms "*visceral gout"* if the urates are deposited in the viscera and "*articular gout"* if the urates are deposited around the joints, which is incorrect. Gout is correctly used as a term in human medicine to describe an enzyme defect that produces an abnormal nitrogenous waste metabolism resulting in uric acid production. In birds, "gout" is a historical misnomer where as urate deposition or hyperuricemia are more correct terms, but for this chapter the historical terms "visceral gout" and "articular gout" have been listed.

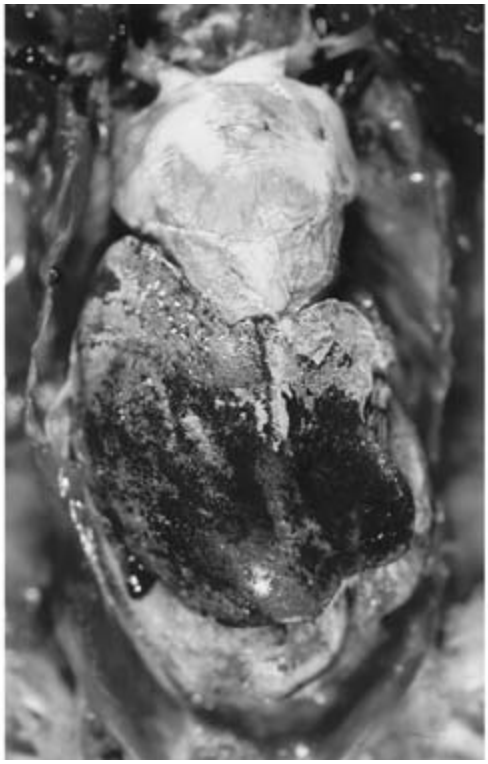
Historically, "gout" occurs as two distinct syndromes, known as visceral urate deposition (or visceral gout) and articular urate deposition (or articular gout). These two syndromes are different from the point of etiology, morphology, and pathogenesis. The following narrative and table (Table 30.1) should help to distinguish between these two entities.

Visceral Urate Deposition ("Visceral Gout")

Visceral urate deposition is a common finding during necropsy of poultry. Visceral urate deposition is characterized by precipitation of urates in the kidneys and on serous surfaces of the heart, liver, mesenteries, air sacs, and/or peritoneum (Fig. 30.27). In severe cases, surfaces of muscles and synovial sheaths of tendons and joints may be involved, and precipitation may occur within the liver, spleen, and other organs. The deposits on serosal surfaces appear grossly as a white chalky coating, while those

Table 30.1. Differences between visceral urate deposition and articular urate deposition in birds (modified from Shivaprasad (422)).

	Visceral urate deposition (Visceral gout)	Articular urate deposition (Articular gout)
Onset	It is usually an acute condition but can be chronic.	It is usually a chronic disease.
Frequency	It is very common.	It is rare or sporadic.
Age	1 day and above.	4–5 months and above. However, immature genetically susceptible chickens may be induced by high protein levels in the diet.
Sex	Both males and females are susceptible.	Mostly males.
Gross lesions		
Kidney	Kidneys are almost always involved, and they look grossly abnormal with deposition of white, chalky precipitates.	Kidneys are normal grossly. Kidneys may become abnormal with white urate deposits if the bird gets dehydrated.
Soft tissues	Visceral organs like liver, myocardium, spleen, or serosal surfaces like pleura, pericardium, air sacs, mesentery, etc. are commonly involved.	Soft tissues other than synovium are rarely involved; however, comb, wattles, and trachea have been observed to be involved.
Joints	Soft tissues around the joints may or may not be involved. Surfaces of muscles, synovial sheaths of tendons, and joints are involved in severe cases.	Soft tissues around the joints are always involved, especially feet. Other joints of the legs, wing, spine, and mandible are also commonly involved.
Microscopic lesions	Generally no inflammatory reaction in synovium or visceral surfaces. Kidney and viscera have inflammatory reaction around tophus.	Granulomatous inflammation in synovium and other tissues.
Pathogenesis	It is generally due to failure of urate excretion (renal failure).	It is probably due to a metabolic defect in the secretion of urates by the kidney tubules.
Causes	1. Dehydration 2. Nephrotoxicity: calcium, mycotoxins, (ochratoxins, oosporein, aflatoxins, etc.), certain antibiotics, heavy metals (lead), ethylene glycol, ethoxyquin, etc. 3. Infectious agents: nephrotropic IBV and avian nephritis virus (chickens), PMV-1 (pigeons), *Eimeria truncata* (geese). In psittacines: *Salmonella* sp., *Yersinia* sp., *Chlamydia psittaci*, microsporidia, cryptosporidia, *Aspergillus* sp., polyomavirus, etc. 4. Vitamin A deficiency. 5. Urolithiasis. 6. Neoplasia (lymphoma, primary renal tumors). 7. Immune mediated glomerulonephritis. 8. Anomalies. 9. Others?	a. Genetics b. High protein in the diet. c. Others?



30.27. Visceral urate deposition over the heart and liver of an adult pigeon.

within viscera may only be recognized microscopically. Much urate is lost when tissues are processed for histology, but evidence of its presence is often seen as blue or pink amorphous material under the microscope. Feathery crystals or basophilic spherical masses may be seen within tissues under the microscope in some cases. Fixing and processing tissues in 90% or absolute alcohol will preserve urates.

Visceral urate deposition is generally due to a failure of urinary excretion. This may be due to obstruction of ureters, renal damage, or dehydration. Dehydration due to water deprivation is a common cause of visceral urate deposition in domestic poultry. Outbreaks of visceral urate deposition in poultry have also been attributed to infectious causes, such as nephrotropic strains of infectious bronchitis virus (80) and renal cryptosporidiosis (462); and noninfectious factors, such as vitamin A deficiency, secondary to urolithiasis (434), treatment with sodium bicarbonate (90), mycotoxins, such as oosporein (355), and feeding growing birds layer rations that are high in calcium and protein (166). It is not clear whether the kidney necrosis seen in visceral urate deposition is primary or secondary to hyperuricemia and urate deposition. In a recent study, one of the first changes observed in birds treated with high concentration of sodium bicarbonate was metabolic alkalosis and hyperuricemia (306). The authors suggested that state of alkalosis induced the breakdown and turnover of nucleoprotein causing hyperuricemia, with the consequent precipitation and crystallization of urates. However, the mechanism for urate crystals precipitation to occur in certain sites is not known, yet.



30.28. Articular urate deposition ("articular gout") in a mature chicken causing enlargement and deformity of toes and feet. (M. C. Peckham)

Articular Urate Deposition ("Articular Gout")

Articular urate deposition, unlike the visceral type, is a sporadic problem of little economic importance in poultry. The clinical condition is characterized by leg shifting, lameness and inability to bend the toes. It is characterized by tophi, deposits of urates around joints, particularly those of the feet, hence confusion with bumble foot. The joints are enlarged and the feet appear deformed (Fig. 30.28). When these joints are opened, the periarticular tissue is white due to urate deposition, and white semi-fluid deposits of urates may be found within the joints. In chronic cases urate precipitates can also be observed in the comb, wattles, trachea, etc. Therapy is palliative only. Removal of the deposits is not recommended due to the difficulty and profuse bleeding. As it has been reproduced by feeding high-protein diets, it is tempting to infer that it results from excess production of uric acid. Studies in a line of chickens bred for high incidence of articular urate deposition, however, indicate that they may have a defect in tubular secretion of uric acid (9, 66).

Urolithiasis

Urolithiasis is primarily seen in laying flocks and has been associated with increased mortality and decreased egg production (273, 490). Urolithiasis is characterized by severe atrophy of one or both kidneys, distended ureters often containing uroliths, and varying degrees of renal and visceral urates deposits.

Clinical Signs and Pathology

Overall mortality in affected flocks may exceed 2% for several months, and in excess of 50% of this mortality may be due to urolithiasis (28, 273). Renal lesions have been recognized in clinically normal birds in flocks undergoing an outbreak, and 3.2–6.3% of hens in some affected flocks had renal lesions at processing (273). Laying chickens die suddenly and may be in good condition and in full lay (28) or they may have a reduced muscle mass, small pale combs, and white pasting on pericloacal

feathers (45). Atrophied kidneys and dilated ureters, often accompanied by diffuse visceral urate deposits, are found in affected birds (28, 45, 273) (Fig. 30.29). The kidney atrophy is often more severe in anterior lobes and is unilateral, but it may be bilateral. The surviving ipsilateral or contralateral lobes may be enlarged. The ureters arising from the atrophied lobes are dilated and full of clear mucus and often contain white irregular concretions or uroliths (45). These uroliths are composed of compact masses of microcrystalline to fine pleomorphic crystals of calcium sodium urate, with random substitution of magnesium and potassium for the calcium and sodium, respectively (327). Microscopic lesions in affected kidneys consist of dilation of ureter branches and tubules, tubular degeneration and loss of tubules, cellular casts, urate crystals, and varying degrees of fibrosis (28, 45). Urolithiasis has been primarily recognized as a disease of laying birds, but reports indicate that lesions and mortality may start during the rearing period (45, 71). In a sequential study of one outbreak, minor focal cortical tubular necrosis was found by microscopic study in grossly normal kidneys of 4-wk-old pullets. In 7-wk-old pullets, the kidneys were grossly swollen with tubular necrosis and casts, eosinophilic globules in glomeruli, and interstitial infiltration of heterophils and lymphocytes. Typical lesions of urolithiasis were found in 14-wk-old birds (45).



30.29. Urolithiasis in a chicken. Severe atrophy of the right kidney and anterior lobes of the left kidney. The right ureter is distended with white material. (Craig Riddell)

Pathogenesis and Etiology

Wideman *et al.* (491) conducted renal function studies on chickens during outbreaks of urolithiasis and concluded that the physiologic impact of the kidney damage was the result of reduced renal mass, rather than of inappropriate renal handling of minerals or electrolytes. A significant reduction in number of glomeruli has also been reported in birds affected by urolithiasis (321). The uroliths may cause sudden death by plugging ureters but probably occur secondary to kidney damage (273). The lesions described in outbreaks of urolithiasis are similar to those described in a long-term study of the pathogenesis of infection of chickens with infectious bronchitis virus (4). In many outbreaks of urolithiasis, it has been difficult to isolate infectious bronchitis virus from affected laying birds (28, 45, 273). This would not be unexpected, as recovery of infectious bronchitis virus was erratic in the long-term study mentioned above. Infectious bronchitis viruses, which have been shown to cause renal damage in experimental chickens, have been isolated in some outbreaks of urolithiasis (45, 71). In several outbreaks, potential problems in vaccination programs against infectious bronchitis have been identified (28, 71, 273).

Excess dietary calcium, in particular if combined with low available dietary phosphorus, fed to growing pullets has caused urolithiasis in experimental trials (151, 416, 479). Exposure of pullets to the Gray strain of infectious bronchitis virus subsequent to feeding a high-calcium laying ration increased the incidence of urolithiasis and gross kidney damage (152). A marked difference in susceptibility to urolithiasis caused by high-calcium diets has been described between two strains of leghorn chickens (262). The more susceptible strain produced more alkaline urine and had a higher proportion of juxtamedullary nephrons (491). Formation of uroliths may be due to high levels of urinary calcium and decreased hydrogen ions in the urine (152). Dietary acidification with ammonium chloride, ammonium sulfate, or methionine has been shown to decrease the incidence of uroliths and gross kidney lesions in urolithiasis induced experimentally with high-calcium diets (150, 262, 487, 489, 488). The use of ammonium chloride was not considered a practical treatment for use in the field because it caused increased water consumption, urine flow, and manure moisture (150). The other compounds did not appear to have this disadvantage (262, 488). Ammonium sulfate was more effective than two forms of methionine in a single trial (262).

Water deprivation has been suggested as a cause of urolithiasis on the basis of field observations (218). The fact that some mycotoxins are nephrotoxic led to the suggestion that they should be considered as a potential cause of urolithiasis (273).

Diseases of the Eye

There are many conditions and diseases that affect the eye, which have been reviewed by Shivaprasad (423).

Ammonia Burn

Ammonia burn describes a keratoconjunctivitis in poultry caused by exposure to ammonia fumes resulting from unsanitary conditions. Ammonia in the poultry house results primarily from the nitrogenous compounds decomposition process occurring in the

litter and fecal matter. Clinical signs include photophobia, excess lacrimation, and respiratory congestion. Affected birds keep their eyelids closed and are reluctant to move. They may rub their head and eyelids against their wings. The cornea has a gray cloudy appearance and may be ulcerated. Edema and hyperemia may be present in the conjunctiva but often may not be very obvious. The condition is generally bilateral and affected birds do not eat and become emaciated. Microscopically, the lesions are characterized by necrosis of the epithelium of the cornea, ulceration, and infiltration of heterophils into the epithelium and substantia propria. The opacification of the cornea is a result of ulceration, cellular infiltration and edema of the cornea (423). Inflammation of the conjunctiva can be severe. Many birds recover if exposure to ammonia fumes is eliminated. Time of recovery depends on the severity of damage to the cornea and may take 1 month or longer if lesions are severe. Prevention of the condition is based on proper ventilation and litter management. The ammonia fumes are formed in wet litter.

Cataracts

The opacification of the lens is referred as cataracts. It is uncommon among most domestic poultry. The condition has been described in chickens, turkeys, and quail. It can be caused by viral diseases, such as avian encephalomyelitis; nutritional deficiencies, such as vitamin E; hereditary, and old age. Lens opacity is normally bilateral, resulting in blindness. Microscopically the lesion is characterized by degeneration of lens fibers, epithelial hyperplasia, formation of bladder cells and liquefaction in advance stages (423).

Developmental Anomalies

Several anomalies are described in young chicks and turkey poults. These include cyclopia, triple eyes, buphthalmia, anophthalmia, microphthalmia, optic nerve hypoplasia, cataracts, retinal dysplasia, corneal edema, and corneal ectasia (128, 145, 371, 419, 423). These defects can have genetic origin, but most are the result of suboptimal incubation conditions. Incubation temperature is one, if not the most, important factor for successful hatching.

Retinal Dysplasia

Abnormal development of the retina most probably inherited as autosomal recessive trait has been described both in layer and broiler commercial chickens (5, 423, 426). The disease is present in chicks when they are a few days old and becomes apparent when they are 5 or 7 days old. Clinically the chicks with retinal dysplasia are smaller than their counterparts, wander aimlessly and are unable to find feed and water. Ophthalmoscopic examination of the eyes in these chicks showed lack of papillary reflexes and normal posterior and anterior segments. The incidence of blindness was generally low, less than 1%, and postmortem examination did not reveal any gross lesions in the eyes. Microscopic examination of the eyes revealed degeneration of photoreceptors (rods and cones) in the earlier stages followed by rosette formation, disorganization of retinal layers, synechiae of the retina, reactive and proliferative retinal pigmented epithelial cells and inflammation of the choroid in later stages. If the birds survived for several weeks progressive changes such as retinal detachment, cataract formation, fibrosis, and metaplastic changes to cartilage were seen (423).

Partial retinal dysplasia and degeneration leading to blindness in 2 to 5% of chickens in a strain of chickens that was a cross between barred Plymouth Rock and Rhode Island red has been described (372). In this flock the clinical signs of blindness were first evident at 5 to 6 weeks of age and by 6 months most of the chicks did not respond to visual stimuli. Retinopathy characterized by loss of photoreceptors resulting in blindness in 0.2% of the chicks in a commercial layer breeder flock has also been described (85). This condition could be diagnosed in chicks as early as 3 weeks of age but it was more evident at 8 weeks of age.

Chorioretinitis and Buphthalmos in Turkeys

A turkey blindness syndrome due to chorioretinitis and buphthalmos has been described in meat (14) as well as breeder turkeys (408). The incidence of eye lesions in turkey breeder flocks ranged from 2 to 30%, and egg production was reduced between 4 and 40%. The affected poults could be recognized by their wandering movement, their tendency to peer at objects in a shortsighted manner, and occasionally by their holding their heads to one side. Blind poults grew normally and were able to locate feed and water. Eyeballs were enlarged by 5–7 wk of age and the corneas flattened. The palpebral fissures became oval. By 16–20 wk of age, many birds had cataracts. Ophthalmoscopic examination of affected eyes revealed pale areas in the retina. On section, severely affected eyes contained an abnormal fluid and some were hard to cut due to bone formation within the eye. Microscopic changes in affected eyes included choroid thickening, degeneration and detachment of the retina, and in severe cases, fibroplasia and islands of ossifying cartilage in the posterior chamber.

Pathogenesis and Etiology

Similar lesions to those just described have been induced in experimental turkeys by rearing them on continual artificial light (8, 14). Rearing experimental chickens under continuous light has caused enlargement of eyeballs, decreased corneal curvature, thinning of the retina and an accumulation of fluid in the vitreous body (255). Birds reared on low intensity, but diurnal, light also develop enlarged eyes, but in such eyes the corneas protrude rather than become flattened (183). Eye enlargement in chickens may also be induced by darkness (210).

Blepharoconjunctivitis

Blepharoconjunctivitis is characterized by inflammation of the eyelids, excess lacrimation, and in severe cases, destruction of the eyeball or inflammation of the eyelids and conjunctiva. This condition has been described in turkeys (20, 21, 334, 406). White frothy foam at the anterior canthus of the eye was followed by accumulation of caseous exudate and swelling of eyelids, which became encrusted and closed. Ulceration of the cornea may result in panophthalmitis and destruction of the eyeball. It has been associated with exposure to *Pasteurella multocida* via drinking

water (334). Previous damage of the eye might have predisposed to infection.

Eye-Notch Syndrome

Eye-notch syndrome refers to a widespread lesion in the eyelid of caged layers (423). The condition appears to start as a small scab or erosion on the lower lid, which develops into a fissure with a tag of flesh attached to one side. Microscopically, it is characterized as blepharoconjunctivitis. The significance and cause of the condition are unknown.

Ophthalmopathy

Cummings *et al.* (83) described this condition in 22-week-old broiler breeders. The condition affected 2 to 3% of the flock. Clinically, birds were partially blind and photophobic. Microscopically, there was retinal degeneration and detachment. Early cataract formation was also noticed. No cause was determined, but low-intensity light for only 6 hours a day might have been responsible.

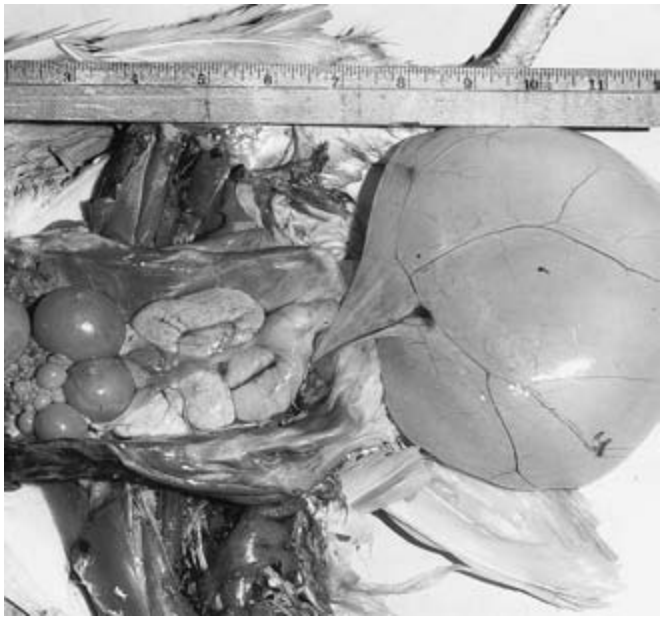
Endophthalmitis

Chronic endophthalmitis of unknown etiology has been described in broiler chickens (493). Grossly it is characterized by opacity of the pupil, cataract formation, retinal thickening and detachment, and shrunken vitreous. Microscopically, there was granulation tissue throughout the eye and atrophy of optic nerves.

Diseases of the Reproductive System

Cystic Right Oviduct

In the female chicken embryo, two Müllerian ducts start to develop into oviducts. The left duct develops into a functional oviduct, while the right duct regresses. If this regression is not complete, partial development will result in a cystic right oviduct. Cystic right oviducts are common incidental findings in postmortem examination of chickens. They vary in size from small, 2-cm diameter, elongated cysts to large fluid-filled sacs up to 10 cm or more in diameter (Fig. 30.30). Small cysts are of little consequence, but large cysts compress the abdominal viscera. The large sacs can result in a bird with a pendulous abdomen and should be differentiated from ascites.



30.30. Cystic right oviduct in a chicken. (M. C. Peckham)

False Layer

The term *false layer* has been used to describe a bird that has the characteristics of a bird in production, visiting the nest regularly but not laying eggs (201). This bird has a normal-appearing ovary and oviduct, but the infundibulum fails to engulf the ovum after it has been ovulated. At necropsy, these birds show excessive amounts of orange-colored fat and have liquid yolk or coagulated yolk in the body cavity. This defect may result as a sequel to infectious bronchitis at an early age (43, 44).

Internal Layer

In some birds, soft-shelled eggs or fully formed eggs may be found in the peritoneal cavity. This indicates that the yolk progressed normally through the oviduct to a certain point and then reverse peristalsis discharged the egg into the body cavity. A bird with a large accumulation of eggs in the peritoneal cavity may assume a penguin-like posture.

Impacted Oviduct

Occasionally, an oviduct is occluded by masses of yolk, coagulated albumen, shell membranes, and in some instances, fully formed eggs. Large masses of yolk-like material may also be found in the oviduct, and upon transection, these masses have the appearance of concentric rings.

Egg-Bound

This term is used to describe a condition in which an egg is lodged in the cloaca but cannot be laid. It may result from inflammation of the oviduct, partial paralysis of the muscles of the oviduct, or production of an egg so large that it is physically impossible for it to be laid. Young pullets laying an unusually large egg are more prone to the problem. Prolapse of the oviduct, usually along with the cloaca, may be seen as a sequela of dystocia.

Phallus Prolapse

It is occasionally seen in anseriformes and ratites associated with infection or immunodepression. The exact cause of prolapse is unknown. In geese, phallus erosions and prolapse have been associated with *Neisseria* spp. infection (13). In ratites usually occurs at the end of the breeding season and after sudden weather changes (54, 193). One report associates *Cryptosporidium* spp. infection with cloacal and phallus prolapse in ostriches (357). Frostbite and bacterial infection may occur as a sequela of phallus prolapse.

Abnormal Eggs and Depressed Production

Poor egg quality and depressed egg production are common problems that cause great economic loss to the poultry industry. They can be due to a multitude of factors involving nutrition, management, environment, and disease.

Diseases of the Integumentary System

Contact Dermatitis

Erosive lesions affecting the skin on the plantar surface of the feet, the posterior surface of the hocks, thigh, or the breast overlying the sternum have been recognized in turkeys and in broiler chickens. Breast, thigh and hock conditions have been an important cause of downgrading of chicken and turkey carcasses (154, 293). Although the incidence in the footpads can be also high, these lesions do not contribute to downgrading of carcasses; but they may result in lameness and depression of body weight (275, 276) and it is becoming an important welfare issue (278). Depending on the site of lesion in the skin lesions have been called by different names. Lesions in the footpads are known as pododermatitis, while lesions on the breast are known as breast burn in chickens (159) and breast buttons in turkeys (154). Ulcers and erosions of the skin covering the thigh and hip of broiler chickens have been described as scabby hip (182, 359). A common feature of all of these skin lesions is that they appear to be due to contact irritation and are associated with poor litter conditions. In recent years, improvements in the litter management and the use of nipple drinkers in the poultry houses have contributed in reducing the incidence of this condition (296).

Clinical Signs and Pathology

Dermatitis on the foot and hock appear as dark black scabs filling ulcers on the plantar footpad, digits and caudal tibiotarsus-tarsometatarsus joint (159, 275). In turkeys, early changes of pododermatitis occur during the first wk of age and become more severe over a short period of time; by 6 wks of age the changes typical of pododermatitis are fully developed (290). Early changes include enlargement of foot scales, cracks, abrasions, and a superficial scab. These changes proceed to a deep ulcer. Histologic lesions include defective keratin in the *stratum intermedium,* particularly adjacent to the ulcer, and infiltration of heterophils in adjacent epidermis. The center of the lesion is occupied by a necrotic mass of cellular debris, which may enclose plant material and bacteria. The base of the mass is underlain by heterophils and often macrophages and a line of giant cells. Many birds also in addition to the foot lesions have similar ulcers filled with black scabs on the posterior of the hock and on the breast.

The breast lesions, in turkeys, were characterized by a granulomatous response with no giant cells noted but connective tissue proliferation occurred below the ulcers (154), probably related to the chronicity of the lesion. Scabby hip syndrome is characterized by ulcers and erosions covered by scabs on the skin of the thigh of broiler chicken associated with femoral head degeneration (359) and in chicken flocks kept in high density (182).

Pathogenesis and Etiology

Field outbreaks of contact dermatitis have been associated with poor litter conditions (159). An epidemiologic study by McIlroy *et al.* (293) found that lesions were more frequent with increased stocking density, increased age, particular feeds, in male birds, and in winter. A study has shown that the decrease in the prevalence of dermatitis in the 90s is associated with the better management of litter and improvements in house designs, particularly the use of nipple drinkers (296) even when stock density and age of slaughter are kept the same as in the 80s. Parameters that were associated to increased prevalence of dermatitis included male only flocks with inferior food conversion ratio and increased mortality rates. A recent study (278) demonstrated that other factors than high commercial stocking densities had more influence in the prevalence of pododermatitis in chickens; while it would probably be necessary to reduce stocking density drastically or to investigate new systems of floor drainage.

In experimental studies, the incidence of dermatitis has been increased by deliberate wetting of litter (174, 275, 276) and by using coarse bedding materials (191, 315). Cool temperatures prior to 12 wk increased the incidence of breast buttons; while, no association was found with the feathering condition (315). Marginal deficiencies of biotin have been suggested as possible cause of pododermatitis in experimental birds (176). In an experiment, the increase of almost ten-fold of biotin in the diet reduced pododermatitis in 19-wk-old turkeys (47). It has been suggested that biotin supplementation in turkey diets should be increased (361).

Breast blisters involving the formation of a subcutaneous cyst between the skin and the sternum (292) should be distinguished from the ulcerative lesions of contact dermatitis in the skin overlying the sternum. Both may be found in the same flock (276), but the breast blisters are more probably due to prolonged pressure from sitting (292) rather than contact irritation. Pressure up to 240mm Hg on the unfeathered breast of turkeys during 6 days for 6 hours daily did not cause any lesions (155).

Xanthomatosis

This condition is rarely seen in poultry today, but it was a significant flock problem circa 1960. It is characterized by an accumulation of semifluid yellowish material under the skin of chickens. Peckham (354) reviewed case reports and studies of the condition. White leghorn hens were primarily affected, and the incidence of affected birds in flocks reached 60%. Birds with lesions were bright, active, and in production. Wattles were often swollen. Swellings also occurred on the breast, abdomen, and feathered portions of the legs. The swellings often became nodular and pendulous. Initially, the lesions were soft and fluctuating and contained a honey-colored fluid. Later, they became firm with chalky white areas of cholesterol interspersed through the abnormal thickened subcutaneous tissue. Histopathologic changes included massive infiltration of foamy macrophages (Fig. 30.31), cholesterol clefts (Fig. 30.32), and giant cells. The cause is unknown, but because the xanthomatous tissue contained high levels of hydrocarbons it was postulated that a hydrocarbon in animal feed may have caused the condition.



30.31. Severe pododermatitis in a 16-wk-old turkey. Bar = 1 cm.



30.32. Histologic section of xanthomatous lesion showing cholesterol clefts. ×470. (AFIP 54-5394)

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Chapter 31

Mycotoxicoses

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Introduction

A mycotoxicosis is a disease caused by a toxic metabolite of a fungus (mycotoxin). Mycotoxins drew attention in the early 1960s when aflatoxin produced by *Aspergillus* spp. was discovered to cause disease in poultry and fish. The disclosure of the carcinogenic properties of aflatoxins accentuated the significance. Diseases in humans and animals caused by moldy food, however, were recognized long before the discovery of aflatoxin. Ergotism, moldy corn poisoning of horses, stachybotryotoxicosis, alimentary toxic aleukia, various hemorrhagic syndromes, yellow rice poisoning, and other acute food poisonings are some of the historically significant mycotoxicoses of humans and animals.

The many naturally occurring mycotoxins now recognized (38, 541) vary by toxicity for poultry, target organs, and occurrence on feedstuffs. The mycotoxins associated with poultry health problems, and therefore the most studied, are the ergot alkaloids, aflatoxins, trichothecenes and other mycotoxins produced by *Fusarium*, ochratoxins, oosporein, citrinin, and fumonisins, but this list is not comprehensive. Although analytical surveys of grains used for poultry feed commonly detect low to moderate levels of mycotoxins, overt intoxication is relatively difficult to document. Despite this, subclinical mycotoxicosis is considered common. The impact of mycotoxins on poultry production may be measured indirectly by the improvements in poultry health that accompany mycotoxin control programs.

Ergotism

Etiology and Toxicology

Ergotism is characterized by vascular, neurologic, and endocrine disorders (reviewed 349). Ergotism descriptions date to the Roman Empire and to China of 5000 years ago, and epidemics killed many people in Europe during the Middle Ages.

Ergotism is caused by *Claviceps* spp., which attack cereal grains. Rye is especially affected, but also wheat and other leading cereal grains, with regional differences. *Claviceps purpurea* is frequently implicated because of its wide host range among cereals. The mycotoxins form in the sclerotium, a visible, hard, dark mass of mycelium that displaces grain tissue. In the normal cycle, the sclerotium falls to the ground, germinates, and produces spores that infect the flower of the new crop, and the cycle repeats. The sclerotium is channeled into the food chain during harvest.

Within the sclerotium are the ergot alkaloids that cause ergotism. Lysergic acid is the chemical building block of the 40 or more alkaloids produced by *Claviceps* spp. With individual variation, the alkaloids produce convulsive and sensory neurologic disorders, vasoconstriction and gangrene of extremities, and altered neuroendocrine control of the anterior pituitary gland (372). Some of the biological activities of the alkaloids are used pharmacologically.

Ergot is detected in wheat, barley, oats, rye, rice, and other cereals in cooler grain-rearing climates worldwide (reviewed 580). Sorghum ergot alkaloids produced by *Claviceps africana* spread globally from Africa in the 1990s (126). Tolerances for ergot in international trade vary, but grain can be declared "ergoty" with sclerotium concentrations as low as 0.1–0.33%. At concentrations that might be encountered naturally (0.33%), ergot has no significant interaction with the antinutritive factors in rye (362, 363). Weed seed contaminants of grain can also be a source of ergot (427). Pelleting feeds can increase the toxicity of ergot, perhaps through increased liberation of toxins.

Natural Disease

Ergotism in poultry reduced feed intake and growth, and caused necrosis of the beak, comb, and toes; and diarrhea. Leghorns developed coalescing vesicles and crusts on the comb and wattles, face, and eyelids (vesicular dermatitis, sod disease) (427). Combs and wattles became permanently atrophied and disfigured. Vesicles and ulcers developed on shanks of the legs and on the toes. In another episode, ergotism spared very young chickens, but those over 6 weeks of age failed to grow and had mortality of 25%. Laying hens had reductions in feed consumption and egg production, but there were no consistent lesions other than on the skin. Wet droppings in laying hens were associated from sorghum ergot produced by *Claviceps africana* (126).

Muscovy ducks fed wheat dockage contaminated with 1.17% ergot became listless and lethargic, stopped eating and drinking, and developed diarrhea (518). Younger ducklings experienced higher mortality than older ducks, with lesions confined to visceral congestion.

Experimental Disease

In chickens, wheat ergot caused reductions in appetite and growth, and mortality, but the effects were quite variable (466, 465, 467). Triticale ergot caused reduction of growth, poor feathering, nervousness, loss of coordination, inability to stand, and

mortality (53). Sorghum ergot impaired weight gain and conversion in broilers (31). Broilers are generally more sensitive to ergot than leghorns. The toxicity is mostly in the alkaloid fraction of the ergot extract, but the value of total alkaloid content is not highly predictive. Ergotamine tartrate, a common alkaloid, caused necrosis of toes in chicks, and cardiac enlargement likely due to increased cardiac workload from vasoconstriction and hypertension (581).

Metabolism and Residues

Ergotamine tartrate accumulated in only trace amounts in broiler tissues when fed at relatively high concentrations (800 mg/kg diet). About 5% of the alkaloid excreted unchanged, and 15–20% as a mixture of metabolites (581).

Fusarium Mycotoxins

The genus *Fusarium* produces many mycotoxins injurious to poultry, including caustic and radiomimetic injury; cardiac toxicity; and skeletal, digestive, and reproductive disorders. In the grain and feed production channels detectable *Fusarium* toxins include trichothecenes, fumonisins, zearalenone and moniliformin, alone or in combinations (481) or with aflatoxin or ochratoxin (7, 181).

Trichothecenes

Etiology and Toxicology

Trichothecene mycotoxins are produced by common soil and plant fungi found worldwide, including *Fusarium* and its perithecial stages, *Calonectria* and *Gibberella*; and the genera *Myrothecium*, *Stachybotrys*, *Cephalosporium*, *Trichoderma*, *Trichothecium*, *Cylindrocarpon*, *Veriticimonosporium*, and *Phomopsis* (reviewed 327, 359, 538). In one study, about 20% of isolates produced trichothecenes. *Fusarium* produces about one-half of the more than 100 trichothecenes (539), with greatest toxin production at high humidity and temperatures of 6–24°C.

Trichothecenes have a tetracyclic sesquiterpene nucleus with a characteristic epoxide ring. Poultry are usually exposed to nonmacrocyclic trichothecenes, which includes type A trichothecenes (T-2 toxin, neosolanial, DAS, and others) and type B (nivalenol, DON, fusarenone-X, and others) (reviewed 327). Toxicity resides in the epoxide ring, which is stable during prolonged storage or normal cooking temperatures (33, 359, 538). In general, trichothecenes damage structural lipids and inhibit the synthesis of protein and DNA (96, 349, 538). Many are caustic irritants, a feature used in detection bioassays.

T-2 toxin, diacetoxyscirpenol (DAS), deoxynivalenol (DON, vomitoxin), and nivalenol occur in feedstuffs worldwide, including corn, wheat, barley, oats, rice, rye, sorghum, safflower seed, mixed feed, and brewer's grains (327, 580). Deoxynivalenol is the most prevalent and can occur naturally with zearalenone, aflatoxin, and other mycotoxins (203, 213, 547). Grains contaminated with DON may be diverted to poultry feeds because DON has low toxicity for poultry compared to swine, which experience feed refusal and emesis.

Natural Disease

Both historical and recent accounts of trichothecene mycotoxicosis reflect caustic and radiomimetic effects expressed as feed refusal, extensive necrosis of oral mucosa and skin in contact with the mold toxins, acute digestive tract disease, and altered bone marrow and immune system function. Recovery generally occurs when an unadulterated diet is provided.

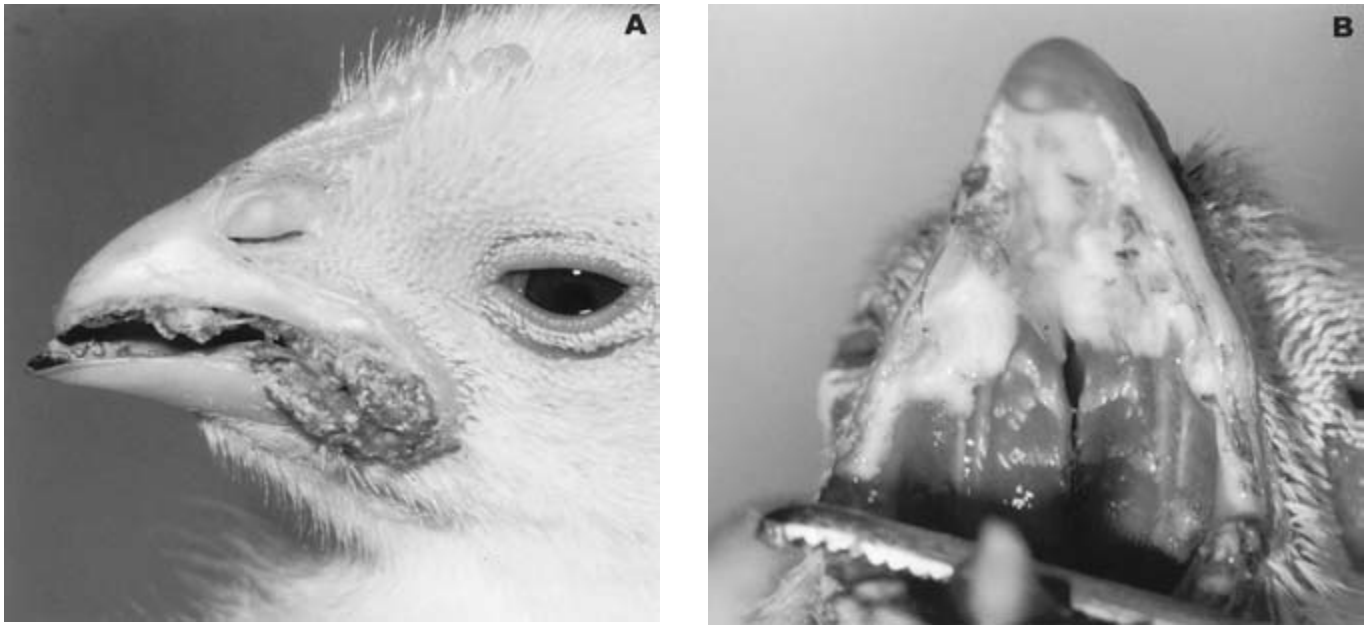
In the premodern era, avian fusariotoxicosis occurred in the former Soviet Union during periods when alimentary toxic aleukia was endemic in humans at certain times during the first half of the twentieth century. *Fusarium poae* and *F. sporotrichioides* isolated from grain and green vegetation feedstuffs were the likely sources of toxins. Chickens with fusariotoxicosis (probable trichothecene mycotoxicosis) had reduced growth, severe depression, and bloody diarrhea (reviewed 313). At necropsy, lesions were necrosis of oral mucosa, reddening of the gastrointestinal mucosa, mottling of the liver, gallbladder distention, atrophy of the spleen, and visceral hemorrhages. Presumed trichothecene mycotoxicosis caused by *Stachybotrys* spp. occurred in poultry during the 1940s as necrosis of oral and crop mucosa, digestive and neurologic disturbances, blood dyscrasias, and hemorrhagic disease (reviewed 237).

More recently in broilers, T-2 toxin produced by *Fusarium tricinctum*-contaminated feed and litter reduced growth and caused skin lesions on the feet and legs, and ulceration and crusting of the oral mucosa (568). Another report found digestive and nervous signs, reduced growth, rickets, abnormal feathering, pigmentation defects, and hemorrhages. T-2 toxin, neosolaniol, verrucarol, fusarenon-X, and crotocol were detected at 1–4 mg/kg in feed produced from crib-stored corn (456).

In hens, feed contaminated with T-2 toxin and HT-2 toxin rapidly decreased egg production beginning the day after feed delivery (485). Depression, recumbency, feed refusal, and cyanosis of the comb and wattles developed, and at necropsy, the ovary and oviduct were atrophied. Feed contaminated with T-2 toxin (3 mg/kg) caused decreases in feed consumption and egg production, and thin-shelled eggs (223). Thick yellow crusts formed over ulcers on the oral mucosa, and feathers were uneven and poorly formed. Oral and feather lesions lacked uniformity within and among cages. At necropsy, hens with oral lesions also had yellow-tan, friable livers, swollen kidneys, urate deposits in the ureters, focal ulceration and inflammation of crop mucosa, and a thickened, rough lining in the gizzard.

Grain sorghum contaminated with DON (0.3 mg/kg) and zearalenone (1.1 mg/kg) was associated with decreased egg production. Oral ulcers occurred in conjunction with squamous metaplasia of salivary and mucous glands (56). Oral crusts and ulcers in commercial layers, taken as presumptive evidence of trichothecene exposure, were associated with decreases in egg weight and shell weight (207). Older hens were more sensitive and the prevalence was influenced by genetic strain.

Deoxynivalenol (190 mg/kg) was the only trichothecene detected in feed associated with egg production losses, oral ulcerations, and gray to black discoloration of the tongue (201). Production returned and oral lesions improved with the addition of an organic aluminosilicate adsorbent to the feed. Deoxyni-



31.1. Fusariotoxicosis. Trichothecene mycotoxins cause chemical irritation of the upper digestive tract mucosa. A. Crusts at the beak commissure of a broiler chicken fed diacetoxyscirpenol for 8 days. B. Beak and palate ulceration and crusting in a broiler chicken following 14 days of consumption of diacetoxyscirpenol (4 mg/kg diet).

valenol did not cause oral lesions, and its presence in the feed was considered an indicator of undetected caustic trichothecenes.

Mortality in Brahma poultry was associated with oral lesions, hemorrhages, and necrosis and depletion in lymphopoietic organs (291). T-2 toxin (0.70 mg/kg) and DAS (0.50 mg/kg) contaminated the feed.

In geese and ducks, barley contaminated with T-2 toxin (25 mg/kg) caused reduced activity, feed refusal, increased water consumption, and death (204, 443). At necropsy, necrosis and pseudomembranes occurred in the esophagus, proventriculus, and gizzard. Histopathology revealed degeneration of intestinal epithelium and acute tubular injury in the kidney. Wild geese dying of fowl cholera were presumed stressed or immunosuppressed by feeding on corn contaminated with DON (< 5.0 mg/kg) and zearalenone (< 25 mg/kg) (236).

In turkey poults, feed refusal and high mortality was associated with feed contaminated with DON (0.81 mg/kg) and salinomycin (2.2 mg/kg) (338). In a feeding trial, much higher concentrations of DON and salinomycin were required to affect feed consumption and cause mortality, leading to the conclusion that undetectable toxins were involved in the incident.

Suspected fusariotoxicosis in sandhill cranes (*Grus canadensis*) was linked to trichothecene-producing *Fusarium* isolated from peanuts used as a food source in the wild (464). Loss of motor control of the neck, wings, and legs accompanied recurring annual episodes of high mortality. At necropsy, edema occurred around the head and neck, but the digestive ulcers usually attributed to trichothecenes were lacking. Principal lesions were hemorrhages, granulomatous myositis, thrombosis, and vascular degeneration.

Experimental Disease

Experimental trichothecene mycotoxicosis in poultry has required several approaches to reproduce fully the disease spectrum observed naturally: purified toxin administered either in solution or in the diet, and toxigenic fungal cultures (reviewed 274). Collectively, these toxins cause feed refusal, impaired growth and reproductive capability; and whole-body pathology including caustic injury to skin and alimentary mucosa; radiomimetic injury to bone marrow, lymphoid tissues, gastrointestinal tract, and feathers; hepatosis; and thyroid alterations.

Neurotoxicity of T-2 toxin and other trichothecenes is inconsistently reported as abnormal wing positioning, seizures, and loss of righting response (242, 571); brain neurotransmitters are affected (95, 517).

Pathology. Many trichothecenes caused erosive and exudative injury to the oral mucosa of poultry fed toxin-appended diets (91, 112, 568, 569). Focal, yellow oral plaques progress to yellow-gray, raised accumulations of exudate with underlying ulcers located near major salivary duct openings on the palate, tongue, and floor of the mouth. Thick crusts accumulate along the interior margin of the beak (Fig. 31.1A,B). Oral histopathology showed mucosal necrosis and ulceration; superficial crusts of exudate, bacterial colonies, and feed components; and submucosal granulation tissue and inflammatory cells.

The histopathology of acute oral intoxication by purified T-2 toxin or DAS was characterized by rapid necrosis and depletion of lymphoid and hematopoietic tissues and then relatively rapid recovery (241). The liver had foci of hepatocyte necrosis and hemorrhage, necrosis and inflammation of the gallbladder mu-



31.2. Feathers from a chicken fed T-2 toxin for 24 days (right) are narrow because of radiomimetic injury to the developing barbs; control (left).

cosa, and then mild proliferation of bile ductules. Necrosis of intestinal epithelium was followed by transient shortening of villi. Necrosis also occurs in the mucosa of the proventriculus and gizzard and in feather epithelium.

Quail were relatively resistant to T-2 toxin (LD50 14.7 mg/kg), but lethal intoxication was associated with necrosis and depletion of lymphoid tissue, and necrosis and fatty degeneration in the liver (209).

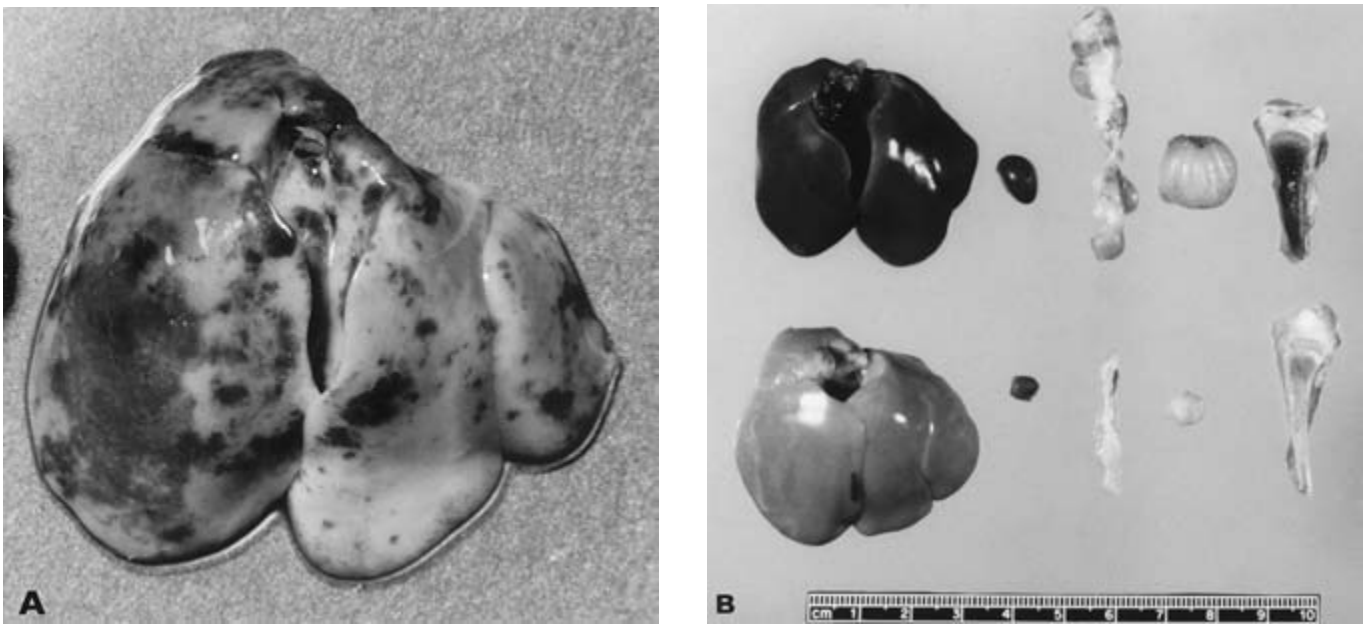
Extended exposure to T-2 toxin and DAS and other scirpenol toxins caused reductions in body weight and skin pigmentation, anemia, and malformed feathers (Fig. 31.2) (244, 243, 416, 572, 575). Lymphoid organs atrophied; the bone marrow became pale red or yellow; and the liver yellows (Fig. 31.3). Histopathology revealed cellular depletion of lymphoid and hematopoietic tissues and in the liver, vacuolar change in hepatocytes and mild proliferation of bile ducts. In the thyroid gland, the follicles became small and had pale colloid.

Deoxynivalenol (DON) is relatively nontoxic to poultry. Feeding DON to broilers produced no clinical signs. Intestinal mucosa development was delayed, with decreased intestinal weight and shortened, narrow villi (22, 23). Feeding DON and zearalenone to ducks caused atrophy of the bursa of Fabricius (124)

Trichothecene-producing cultures of *Fusarium* and *Stachybotrys* caused clinical signs and lesions similar to those of the purified toxins (242, 273, 480). Trichothecenes in fungal cultures, a model closer to natural intoxication, appeared more toxic than purified compounds. This is suggestive that even at a low concentration, certain trichothecenes are significant if detected in feedstuffs.

Mallard and Muscovy ducks were particularly sensitive to trichothecenes and develop extensive lesions in the oral cavity and gastric organs, as well as atrophy of the lymphoid organs (228, 376, 487).

Acute lethal DON intoxication in broiler chickens caused reduced spontaneous activity, dyspnea, diarrhea, visceral urate deposition (visceral gout), and hemorrhages in the subcutis and viscera (225). Broiler and leghorn chicks, leghorn hens, and turkey poults tolerated DON at levels likely to be encountered under natural exposure (39, 40, 220, 265, 286, 299). Oral plaques and gizzard erosions developed but only at concentrations much higher than other trichothecenes and greater than those causing feed refusal and emesis in swine (334, 365).



31.3. Experimental trichothecene mycotoxicosis in broiler chickens. A. Hemorrhage in the liver occurring 20 hours after consumption of feed mixed with a culture of *Fusarium sporotrichiella* that produced T-2 toxin and neosolaniol (242). B. Daily oral doses of T-2 toxin caused yellow discoloration of the liver (bottom row) compared with control (top row). The radiomimetic effects are evidenced by atrophy of the spleen, thymus, and bursa of Fabricius and yellow discoloration of the bone marrow (243).

Chronic nivalenol intoxication of leghorn hens reduced feed intake but not egg production. At necropsy after 55 days, lower doses caused pale fragile liver, and higher doses caused lesions in the gizzard, hemorrhages in the duodenum, and swelling of the cloaca and oviduct (182).

Clinical Pathology. In broilers, T-2 toxin and DAS generally caused anemia associated with marked hematopoietic depletion in the bone marrow (242, 243, 412). In laying hens, T-2 toxin caused leukopenia and DON caused mild anemia and leukopenia (299, 573).

Broilers fed growth-inhibitory levels of T-2 toxin impaired blood coagulation (134, 136). Serum biochemical tests in chickens and quail reflected lesions in the liver, intestine, muscle, and kidney, which recovered to normal within 10 days (89, 90, 209, 420; 575).

T-2 toxin reduced plasma vitamin E concentrations in broilers (105), likely through impaired lipid metabolism in the intestine.

Reproduction and Egg Production. Trichothecenes effect egg production and reproduction. T-2 toxin, DAS, and monoacetoxyscirpenol reduce feed intake, body weight, and egg production in leghorns, broiler breeders, and turkey hens (10, 11, 54, 90, 460, 575). T-2 toxin and DAS had synergistic effects on egg production (129). Decreases in egg production were abrupt, and intoxication impaired hatchability. During recovery, hens overconsumed feed (575). Short-term DAS intoxication, however, had little effect on broiler breeder egg production (55). Deoxynivalenol was essentially nontoxic to hens at concentrations likely to be encountered naturally (39, 220, 221, 286, 301, 334, 367). Mild changes in dietary intake were due to palatability or olfactory responses, and there were minor changes in egg components and embryonic mortality.

Immunosuppression. T-2 toxin fed to broilers decreased the splenic lymphocyte stimulation index, and hemagglutination inhibition titers to Newcastle virus (282). Despite the profound effects of many trichothecenes on lymphoid organs and bone marrow (522), measurable immunosuppression is not well documented in chickens or turkeys (50, 457, 494). This may result from using purified toxin-appended diets rather than feeding fungal cultures or oral dosing, which allow greater expression of toxicity by trichothecenes (242).

Ducklings fed T-2 toxin had lymphocyte depletion in the bursa of Fabricius, thymus, and spleen, and reductions in the lymphocyte mitogenic responses (266, 448). T-2 tetraol was cytotoxic to chicken macrophages *in vitro* (288).

Pharmacological Interactions

T-2 toxin reduced the anticoccidial activity of lasalocid (544).

Metabolism and Residues

Liver chiefly metabolized and excreted T-2 toxin in chickens (93). After a single exposure, only T-2 toxin was detected in liver, but T-2 toxin, HT-2 toxin, neosolaniol, T-2 tetraol, and others appeared in feces (196, 579), and most excreted from the body within 48 hours (463). Relatively small amounts of T-2 toxin excreted into the egg (92), detectable in yolk and albumen.

Deoxynivalenol fed to broilers at field levels was undetectable in skeletal muscle (159). The concentration in hen plasma reached only 1% of an oral dose, and was rapidly eliminated in feces (437). Deoxynivalenol transmitted to eggs at trace to undetectable concentrations (543, 519). Nivalenol fed to hens occurred unchanged and in trace amounts in bile, and excreted to feces with a related metabolite (182).

Moniliformin

Etiology and Toxicology

Moniliformin, produced by *Fusarium verticillioides* (formerly *F. moniliforme*) and other *Fusarium* spp. (447), is cardiotoxic and nephrotoxic in poultry. *F. verticillioides* causes ear rot, kernel rot, and stalk rot of unharvested corn and occurs in stored high-moisture shelled corn and on oats, soybeans, sorghum, barley, wheat, and corn that are visually sound. Although moniliformin is quite toxic to poultry, diets made with purified moniliformin are less toxic than toxigenic fungal cultures. *F. verticillioides* also produces fumonisins, zearalenone, fusariocin A, and other toxic fractions (71).

Natural Disease

Verification of *F. verticillioides* (*F. moniliforme*) mycotoxicosis is relatively lacking, although industry reports indicate that it is a problem for poultry. Corn contaminated with *F. verticillioides* reduced rate of lay and delayed peaks in production in broiler breeders and leghorns (113). Intermittent overconsumption and underconsumption of feed occurred with diarrhea, dark fecal droppings with undigested feed, fecal-stained eggshells, and blood smears on eggshells. Contaminated corn was high in moisture, low in protein, and high in crushing strength, which caused large particle sizes in the feed leading to maldigestion.

Experimental Disease

Moniliformin toxicity is similar in the chicken, turkey, quail and duck and causes cardiac toxicity, ascites, and nephrosis. Intoxication by purified moniliformin caused reductions of feed intake and weight gain, slowing of the heart rate, dyspnea and cyanosis. In combination with fumonisin, moniliformin caused sudden death that resembled spiking mortality of broilers, with decreased blood glucose (270, 271, 453). Moniliformin is more toxic than fumonisin and the interactive effects were additive (324). Hens tolerated moniliformin at concentrations toxic to younger birds (311).

Lesions at necropsy were enlargement of the heart, ascites, and digestive and cutaneous hemorrhages and edema (41, 106, 166, 226, 374, 548, 583). The heart developed various degeneration progressing to necrosis of the cardiac muscle (44, 45, 226, 369), which was partially alleviated by selenium. Kidney had nephrosis with mineralized casts. Liver developed vacuolation, swelling, and focal necrosis of hepatocytes; bile duct proliferation and fibrosis occurred with chronic intoxication.

Antibody to Newcastle disease vaccination was decreased, as were serum immunoglobulins and macrophage activity when in combination with fumonisin (329, 330, 446).



31.4. Diarrhea (A) and catarrhal enteritis (B) in broiler chickens fed cultures of *Fusarium moniliforme* that produced fumonisin B1.

Fumonisins

Etiology and Toxicology

F. verticillioides (*F. moniliforme*) also produces the fumonisins, which are the cause of equine leukoencephalomalacia (moldy corn poisoning) (343) and porcine pulmonary edema syndrome (109). Several fumonisins are produced (B1, B2, B3), but fumonisin B1 is the most common. Other species of *Fusarium* also produce fumonisins (167, 233). The toxicity of fumonisin B1 relates to disrupted sphingolipid synthesis (555).

Natural Disease

Fumonisin and aflatoxin contamination of feed for laying hens caused black adhesive diarrhea, reductions in feed intake, egg production, and body weight, lameness, and increased mortality (436). The diarrhea was reproduced in chicks and in laying hens with the suspect feed and with diets spiked with fumonisin.

Experimental Disease

Fumonisin B1 caused diarrhea, catarrhal enteritis (Fig. 31.4A, B), and impaired weight gain and feed conversion in turkey poults, broiler chicks, and ducklings (42, 59, 63, 167, 232, 307, 308, 309, 322, 556). Poults are more sensitive than chicks, but poultry are quite resistant to fumonisins in comparison to horses and swine. Although concentrations toxic to poultry are much higher than those likely to occur in grain (306), *F. moniliforme* produces other toxins that could influence the safety of these feedstuffs for poultry (557).

Lesions of experimental toxicity were consistent enlargement of the liver and variable enlargement of the kidney, pancreas, and proventriculus and gizzard; atrophy of lymphoid organs; and rickets. Histologically, the liver had multifocal necrosis of hepatocytes, hyperplasia of hepatocytes and bile ductules, and hypertrophy of Kupffer cells (43,44, 125). The intestine had villous atrophy and goblet cell hyperplasia. Rickets developed with growth plates widened in both the zones of proliferating and hypertrophied cartilage. Fumonisin B1 in combination with moniliformin caused lesions reflective of both toxins, including ascites, and enlargement of the heart, liver, kidney, and lung (272). Immune system changes included lymphoid depletion from the thymus, decreased mitogenic responses, decreased bacterial clearance, and toxicity to macrophages and lymphocytes (86, 140, 285, 329, 330, 445). Hemostasis and serum proteins were mildly affected (168).

Increased sphinganine:sphingosine ratios occurred in the liver with fumonisin B1 toxicity in chickens and ducks (42, 232). This parameter is a potential indicator of fumonisin B1 toxicity in humans, and ducks have become a model for studying this marker of exposure (527). Fumonisin B1 is metabolized by the liver and excreted in feces(125).

Hens tolerated relatively high concentrations of fumonisin B1 with only transient adverse effects during the laying cycle (311).

Quail fed fumonisin B1 as diets appended with cultures of *F. verticillioides* had intoxication of variable severity including mortality (21, 74, 127, 387). Reductions in growth rate and egg

production, and increased susceptibility to *Salmonella* infection occurred with chronic hepatotoxicity with bile duct proliferation.

Fusarochromanone

Etiology and Toxicology

Fusarium spp. also produce fusarochromanone, which causes tibial dyschondroplasia (TD) in chickens. Long bone deformities in broiler chicken have been induced by cultures of *F. moniliforme* and *F. roseum*, *F. equiseti* as well as *Aspergillus niger* and *A. flavus* (100, 342). *F. roseum* isolated from overwintered barley in Alaska caused tibial dyschondroplasia when fed to broilers (551). Defective chondroclasis was a possible pathogenic mechanism. Of six components identified, one of the fluorescent components, TDP-1 (fusarochromanone), induced a 100% incidence of TD when fed to broilers (325, 326).

Experimental Disease

Chicks fed fusarochromanone as cultures of *Fusarium* developed dyschondroplasia in the tibial growth plate in 4 days (229). Although the action of fusarochromanone at the cellular and biochemical level is unknown, increased dietary copper and zinc had a partial sparing effect (565). The hypertrophic cartilage had a lower density of chondroclasts (319), and the chondrocytes from the cartilage core have degenerative changes that were likely secondary to the increased distance from their vascular supply (230). Fusarochromanone was much less toxic than T-2 toxin to chondrocytes *in vitro* (564). *Fusarium* strains that produced fusarochromanone were also immunosuppressive (565, 566).

Zearalenone

Etiology and Toxicology

Grains infected with the fungus *Gibberella zeae* (*Fusarium graminearum*, *F. roseum* Graminearum) are a source of zearalenone, a mycotoxin with estrogenic activity. Of seven chemical forms, only zearalenone and zearalenol occur naturally. Although zearalenone is the most prevalent and most studied in poultry, zearalenol is more active estrogenically (360). Zearalenone occurs in corn, sorghum (114), wheat, barley, oats, milo, rye, and other grains (reviewed 489, 580). Toxicity occurs chiefly in swine as reproductive failure. Chickens tolerate zearalenone better than turkeys or swine and provide an outlet for grains unfit for swine (6, 170). Zearalenone is relatively nontoxic for chickens, but it has potential adverse effects and may be an indicator of other potentially toxins present.

Natural Disease

Zearalenone (0.5–5.0 mg/kg) was detrimental to broiler breeders that experienced a reduction in egg production; however, fertility, hatchability, or broiler performance remained normal (49). The hens had lowered serum progesterone, ascites, and cystic inflammation of the oviduct.

Experimental Disease

Studies of zearalenone mycotoxicosis in broiler and leghorn chickens, turkeys, quail, and geese indicate relative resistance compared to swine. Turkeys are the most sensitive, with reproductive tract and sex hormone-sensitive tissues targeted. Japanese quail are resistant (28).

In leghorn chicks, bursa of Fabricius weight increased (94, 504,) possibly related to hormone-induced cloacal swelling in birds. Cysts developed on the peritoneal surface and within the oviduct. Broilers were highly tolerant of zearalenone, with lesions limited to decreased comb and testes weight (9), oviduct enlargement (94), and leukopenia. Male turkey poults displayed precocious strutting behavior and developed caruncles, dewlaps, and soft tissue swelling of the vent (8).

In leghorn hens, egg specific gravity, eggshell thickness, and interior egg quality were reduced (504). Serum calcium decreased and phosphorus increased (94). Feed contaminated with zearalenone and DON reduced feed intake and egg production (122). Other studies showed leghorns highly tolerant of zearalenone and of corn contaminated with *F. roseum* (6, 94, 345). A water-soluble component of *F. roseum* cultures containing neither zearalenone nor trichothecenes caused reduced hatchability (325). In geese, fertility was reduced and spermatogenesis was inhibited (409, 410, 411). Turkey eggs had reduced hatchability, but neither zearalenone nor trichothecenes were the responsible toxins (10).

Metabolism and Residues

Zearalenone was distributed chiefly to liver and gallbladder (361) and excreted in feces as zearalenone and zearalenol (394). Egg residues occurred only in yolk (119) or were not detected (122, 519).

Other Fusarium *Toxins*

Strains of *F. moniliforme* (now *F. verticillioides*) that produced fusaric acid (101) and fusarocin C (344) were immunosuppressive in chickens. Fusaric acid, although a mild toxin, was synergistic with fumonisin B1 in toxicity in a chick embryo assay (29).

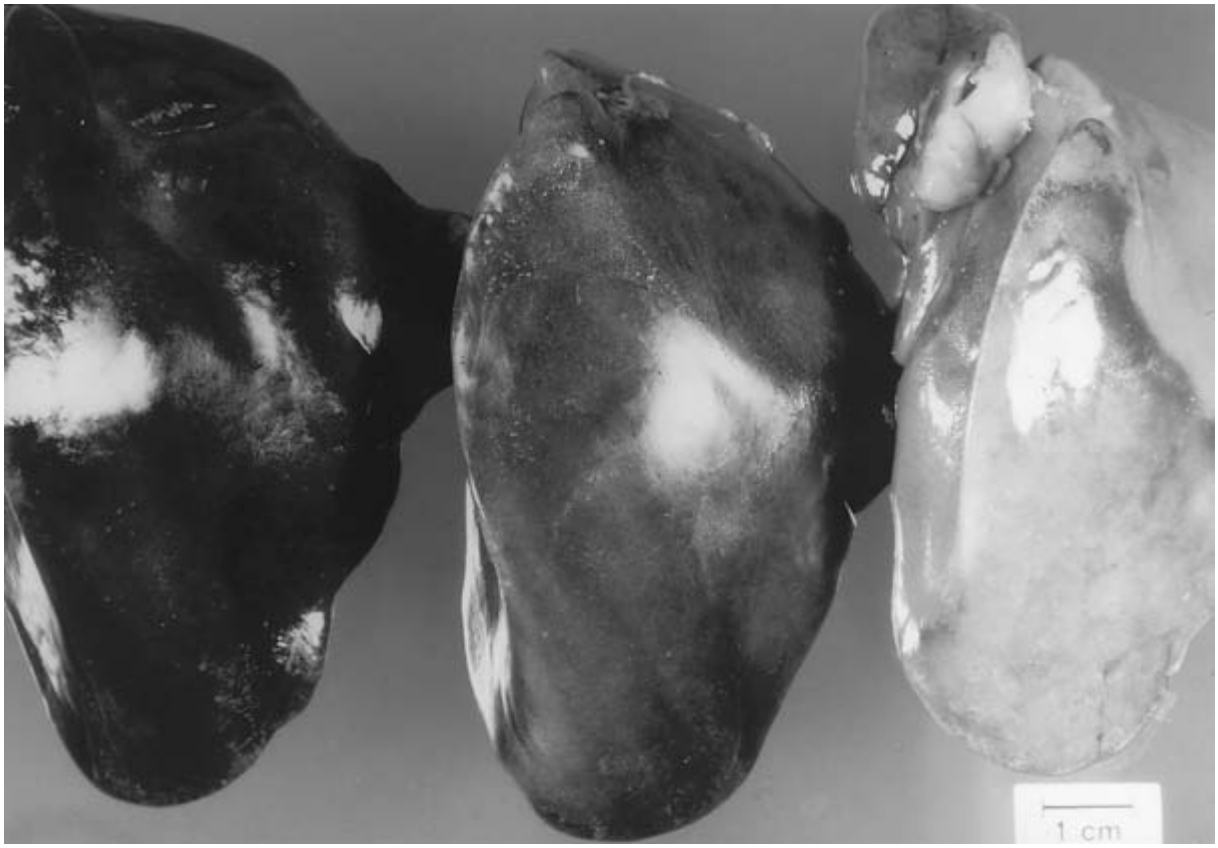
Chicks fed *F. moniliforme* developed signs of thiamin deficiency and responded to thiamin therapy (174). Dietary thiamin concentrations were low, possibly due to the thiamin destruction or utilization by the mold in the feed. Fumonisin B1 contaminated feed associated with paralysis in quail, but clinical signs could not be reproduced in feeding trials (208). Aurofusarin produced by *F. graminearum* deteriorated egg quality (350).

Aflatoxins

Etiology and Toxicology

Aflatoxins are highly toxic and carcinogenic mycotoxins produced by *Aspergillus flavus*, *A. parasiticus*, and *Penicillium puberulum* (reviewed 157). Poultry feeds and ingredients are vulnerable to fungal growth and aflatoxin formation. Aflatoxins are relatively stable in normal food and feed products but are sensitive to oxidizing agents such as hypochlorite (commercial bleach).

The aflatoxins have two fused dihydrofuran rings with various moieties, and members are designated by their blue (B) or green (G) color reaction to fluorescent light and their chromatographic Rf values. Aflatoxin B1 is the most toxic, and hepatotoxicity is the primary effect in nearly all animals. Chronic aflatoxicosis



31.5. Lethal aflatoxicosis in turkeys caused liver discoloration from dark red (left), due to congestion and necrosis, to yellow (right), owing to fat accumulation in hepatocytes. Aflatoxin B1 (200 ppb) was detected in the feed.

results in neoplasia in many species, usually in the liver, but gallbladder, pancreas, urinary tract, and bone may be involved (reviewed 408). Although several aflatoxin metabolites are carcinogenic, aflatoxin B1 is most potent. It binds to nuclear and mitochondrial DNA and is a model hepatocarcinogen for mechanisms of tumor initiation in the liver (reviewed 250). It may also be active in promotion through oncogene activation, hormone alteration, and dietary interaction.

Aflatoxin-producing fungi and aflatoxin-contaminated animal feedstuffs are recognized worldwide (115, 245, 287, 383, 484, 489, 580), usually with adverse implications for poultry production (275, 276, 337, 368).

Natural Disease

Previous editions provide detailed descriptions of the first cases of aflatoxicosis (247, 422). Early accounts of aflatoxicosis in literature are now recognized to have significant contributions from cyclopiazonic acid and possibly sterigmatocystin and other toxins (52).

Lethal aflatoxicosis in ducklings occurred as inappetance, reduced growth (20), abnormal vocalizations, feather picking, purple discoloration of legs and feet, and lameness. Ataxia, convulsions, and opisthotonus preceded death. At necropsy, liver and kidneys were enlarged and pale. Chronic cases had hydropericardium and ascites, shrunken firm nodular liver, bile-distended gallbladder, and hemorrhages. Microscopic lesions in the liver were fatty change in hepatocytes, proliferation of bile ductules, and extensive fibrosis, accompanied by vascular and degenerative lesions in pancreas and kidney.

Turkeys developed inappetance, reduced spontaneous activity, unsteady gait, recumbency, anemia, and death (491, 552). At necropsy, the body condition was generally good, but there was generalized congestion and edema. The liver and kidney were congested, enlarged, and firm; the gallbladder was full; and the duodenum had mucoid content. Lethal aflatoxicosis caused either dark red or yellow discoloration of the liver due to congestion or fat accumulation, respectively (Fig. 31.5). Microscopic lesions in livers were swollen hepatocytes with homogenous to vacuolated cytoplasm, karyomegaly, and focal necrosis of centrilobular hepatocytes. Chronic cases had hepatocyte regeneration, proliferation of bile ductules, and reticuloendothelial cell hyperplasia and degenerative lesions in the heart, kidney, and intestine.

Aflatoxicosis in chickens closely resembled that in ducks and turkeys (19, 20). The occurrence of skeletal myopathy (184) may have reflected an interaction of selenium and aflatoxicosis (see "Experimental Disease").

Aflatoxicosis occurs in poultry worldwide (4, 60, 98, 116, 214, 234, 281, 286, 314, 391, 435, 452, 451, 490, 498). The direct and indirect effects of aflatoxicosis include increased mortality from

heat stress (broiler breeders) (116); loss of egg production (leghorns) (66); anemia, hemorrhages, liver condemnations (314), paralysis, lameness (391), and impaired performance of broilers (276, 454); nervous signs (4), and mortality (ducks) (66); impaired ambulation and paralysis (quail) (561); impaired immunization (turkeys) (231); and increased susceptibility to infectious disease in many species (66, 435). Cases of concurrent aspergillosis and aflatoxicosis confirmed that *Aspergillus* spp. threaten poultry production in the feed, litter, and environment (451, 490).

Experimental Disease

Aflatoxicosis impairs all important production parameters including weight gain, feed intake, feed conversion efficiency, pigmentation, processing yield, egg production, and male and female reproductive performance. Some influences are direct effects of intoxication, and others are indirect, such as from reduced feed intake.

Susceptibility of poultry to aflatoxins varies among species, breeds, and genetic lines. In general, ducklings, turkeys, and pheasants are susceptible; and chickens, bobwhite and Japanese quail, chukar partridge, and guinea fowl are relatively resistant (17, 210, 213, 264, 332, 473). Wide variation exists among breeds, with age and sex also being important (65, 110, 559).

Pathology

The pathology of experimental aflatoxicosis is similar to the naturally occurring disease. Acute intoxication in ducks caused pale, yellow-green discoloration, and atrophy of the liver, with the left lobe being more affected (380). Microscopic lesions chiefly involved hepatocytes as cytoplasmic vacuolation (fatty change) and massive necrosis, often accompanied by hemorrhage. Proliferation of bile ductules developed by the second day and progressed rapidly. Subacute lethal intoxication of ducks, especially those fed cultures of *A. flavus*, caused extensive necrosis and loss of hepatocytes and severe proliferation of bile ductules. In nonlethal aflatoxicosis, the liver had fatty change in hepatocytes, karyomegaly, numerous mitotic figures, and proliferation of bile ductules (246). Membranous glomerular lesions and interstitial fibrosis occurred in the kidneys of ducks and goslings (373). Figures 31.6A–C and 31.7A–C illustrate the histologic changes in liver and kidney.

Aflatoxicosis in chickens caused yellow, ocher discoloration of the liver, with multifocal hemorrhage and a reticulated pattern on the capsular surface. In time, the livers developed white foci as hepatic lipid content increased. Histologic lesions occurred as fatty vacuolation of hepatocyte cytoplasm; karyomegaly and prominent nucleoli in hepatocytes; proliferation of bile ducts; and fibrosis. Basophilic, vacuolated, regenerative hepatocytes, and inflammation by heterophils and mononuclear cells occurred in the portal zones (77, 246). Turkeys developed bile duct proliferation and nodular regeneration of densely eosinophilic hepatocytes that compressed adjacent parenchyma (246, 381). Vacuolar change and fibrosis were mild, even in turkeys that died following prolonged toxin ingestion. For these studies in chickens, no aflatoxin-related lesions were reported in either the kidney or major lymphoid tissues (373).

Clinical Pathology

Aflatoxin caused anemia characterized by reductions in the packed cell volume, erythrocyte count, hemoglobin concentration, and mean corpuscular volume (261, 317, 364). Iron absorption and retention initially decreased but then normalized (316). Young birds were more susceptible to anemia (317). Total leukocytes were increased, but with concurrent lymphopenia (317, 531).

Aflatoxin decreased total serum protein, lipoprotein, carotenoid pigment, cholesterol, triglycerides, uric acid, calcium, phosphorus, iron, copper, zinc, and lactate dehydrogenase (139, 171, 261, 532, 540). Serum sorbital dehydrogenase, glutamic dehydrogenase, and potassium were increased (117, 444). Ducklings were similar(379). In selected lines of Japanese quail, the degree of reduction in total protein and albumin and the degree of increase in ß-glucuronidase correlated with resistance to aflatoxin (426). Blood clotting time and ratio of aspartate aminotransferase to alanine aminotransferase were indicators of resistance in ducks (402).

Bruising is a problem during transport and slaughter of poultry. Aflatoxin promoted bruising by increased capillary fragility and reduced shear strength of skeletal muscle (401). It also impaired coagulation in chickens and turkeys by interfering with several coagulation components, notably prothrombin (138, 135, 530, 562). Aflatoxin altered coagulation more than either ochratoxin A or T-2 toxin, but the effects of ochratoxin A lasted longer (135, 260).

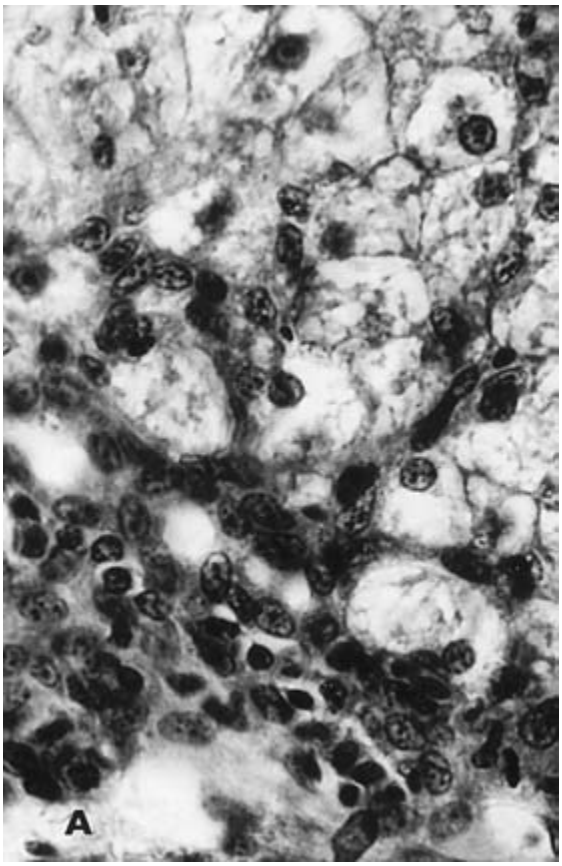
Nutrition and Digestion

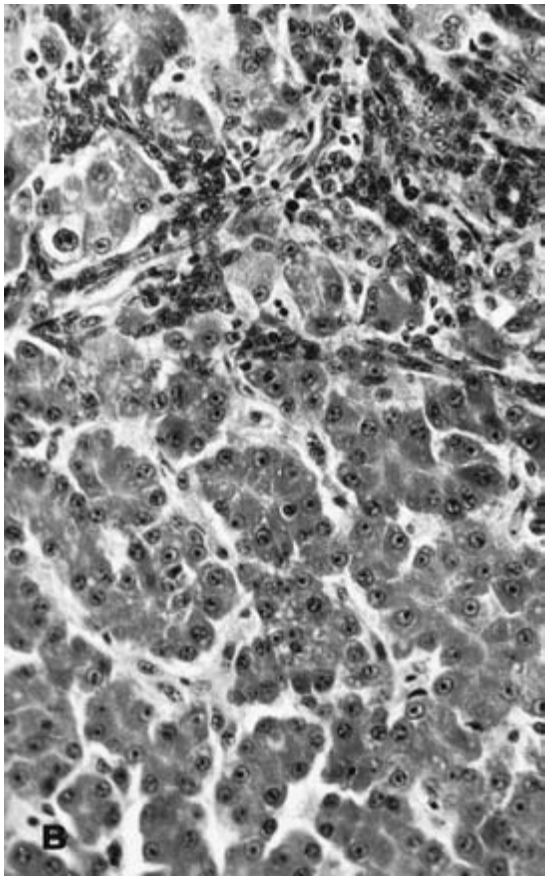
Skin pigmentation is desirable for broilers in some markets. Aflatoxin impaired pigmentation in broilers by inhibiting intestinal absorption of carotenoid pigments and causing sequestration in the liver (476, 477, 534, 533, 535). The severity of aflatoxicosis was enhanced by a diet low in fat (216, 459), protein (401, 460), and riboflavin or vitamin D3 (215, 217), and by a diet high in tannic acid (121). Aflatoxin influenced calcium and phosphorus metabolism by altering the metabolism of vitamin D and parathyroid hormone (198); however, conclusions regarding the need for supplemental vitamin D3 in aflatoxicosis are equivocal (47, 58). In broilers, a deficiency of pancreatic amylase and lipase resulted in steatorrhea (399, 400), but laying hens were resistant. These variables make it difficult to establish minimum- or no-effect concentrations for aflatoxin in poultry feeds.

Reproduction and Egg Production

Mature Leghorn males experienced lowered semen volume, testis weight, and spermatocrits, and testosterone values, caused indirectly by reduced feed intake during aflatoxicosis (482, 483). Microscopic examination of sperm revealed abnormal spermatozoa and histologically, a cessation spermatogenesis in seminiferous tubules (396). Plasma testosterone concentration and responsiveness were lowered in maturing leghorn males (103). Broiler breeder males had reduced body weight and mild anemia but semen was unaffected (57, 570).

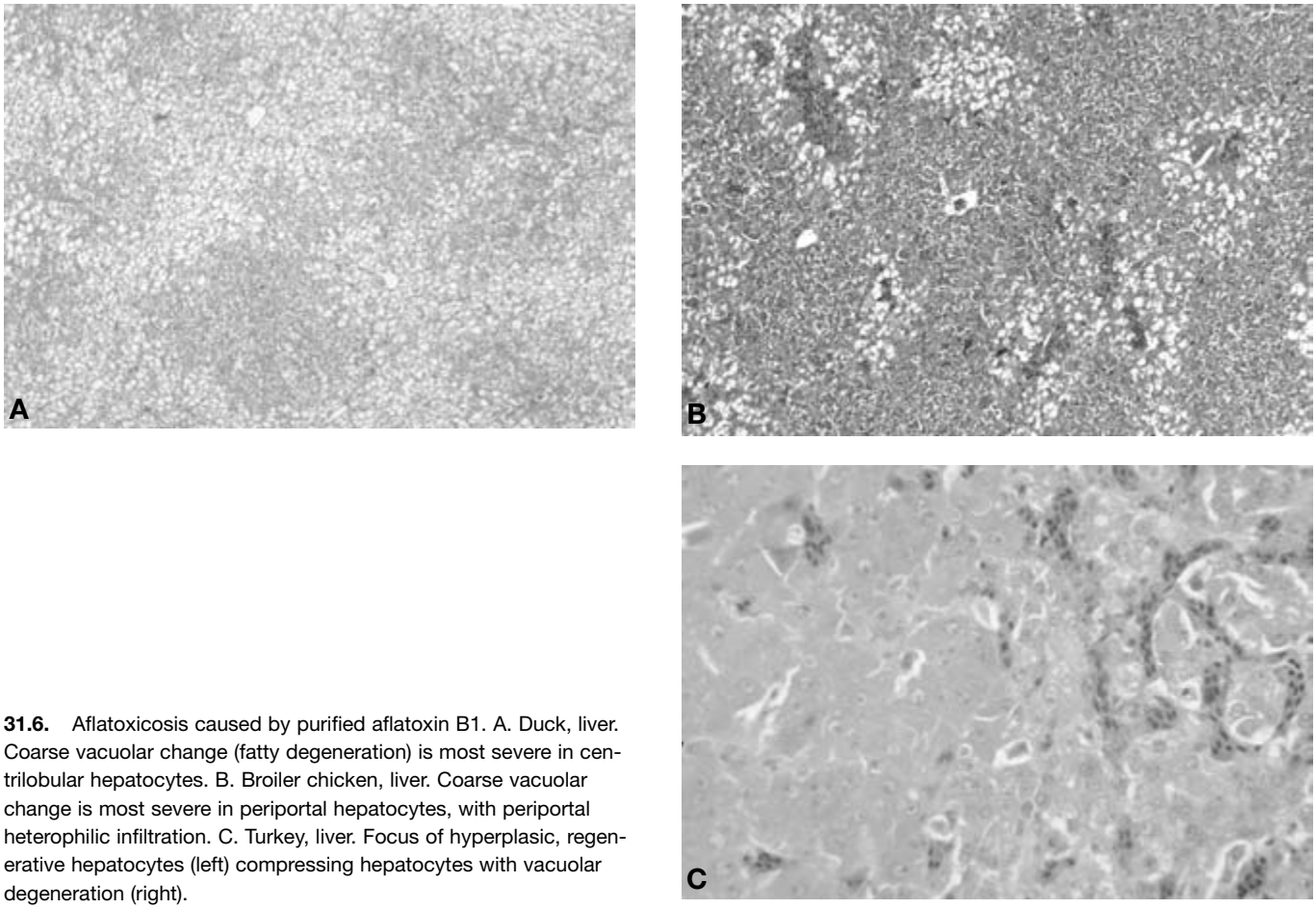
Loss of hatchability due to embryonic death was the most sensitive indicator of aflatoxicosis in broiler breeders and leghorns (111, 249), and was more sensitive than egg production (293).







31.6. Aflatoxicosis in ducks fed toxic peanut meal. A. Early liver lesion showing degenerative changes in parenchyma and bile duct hyperplasia. B. Nodular hyperplasia of liver parenchyma and bile duct hyperplasia are present. C. Kidney. Proximal tubules are dilated; epithelium is undergoing necrosis; and some nuclei have enlarged bizarre forms with prominent nucleoli (arrow).



31.6. Aflatoxicosis caused by purified aflatoxin B1. A. Duck, liver. Coarse vacuolar change (fatty degeneration) is most severe in centrilobular hepatocytes. B. Broiler chicken, liver. Coarse vacuolar change is most severe in periportal hepatocytes, with periportal heterophilic infiltration. C. Turkey, liver. Focus of hyperplasic, regenerative hepatocytes (left) compressing hepatocytes with vacuolar degeneration (right).

Egg production was spared despite mild hepatotoxicity, although once declined may require several weeks to return (169, 185, 267). Aflatoxin impaired egg production by reducing synthesis and transport of yolk precursors in the liver, with egg size and yolk parameters decreased (254). In Japanese quail, aflatoxin hepatotoxcity impaired of feed conversion, delayed maturation, and reduced egg production and egg quality (137, 405, 474, 393, 404).

Immunosuppression

Spontaneous aflatoxicosis is strongly associated with increased susceptibility to infectious disease (156, 430, 452). Experimental definition of immunosuppression is less conclusive and reflects the complexity of defining mechanisms of natural toxins. Experimental approaches include purified toxin alone or in combinations, crude cultures or culture extracts of a cloned fungal species, and naturally contaminated feeds or grains. Potential interactions of mycotoxins and dietary nutrients possibly influence the results and definition of an effect at a given concentration.

In chickens, aflatoxin increased susceptibility to, or severity of, cecal coccidiosis, Marek's disease (155), salmonellosis (497, 574), inclusion body hepatitis (471, 492), and infectious bursal disease virus (85, 501). Vaccination failures were linked to aflatoxicosis in chickens (14, 36, 442), and impaired response to vaccination responses was demonstrated for Newcastle disease, infectious bronchitis, infectious bursal disease, and fowl cholera (25, 26, 67, 178). Turkeys experienced vaccination failure to fowl cholera and increased susceptibility to coccidiosis (428, 563).

Aflatoxin-induced immunosuppression accompanied atrophy of the bursa of Fabricius, thymus, and spleen (85, 429), regardless of the immune response genetics (536, 537). Aflatoxin was toxic for B lymphocytes in the late-stage embryo, and immune dysfunction was identified in progeny of broiler breeders exposed to aflatoxin (434, 445). In ducks, lesions in the lymphatic system accompanied abnormalities in circulating lymphocytes (495), lymphocyte depletion from the thymus, and reduced mitogenic responses of B and T lymphocytes (266). Clearance functions of blood phagocytes and the reticuloendothelial system were impaired (80, 79, 81, 357, 364), and serum complement activity was reduced in chickens (508). Cell-mediated immune responses decreased in both turkeys and chickens (192, 191, 190). Despite the previous explanations of aflatoxin-induced immunosuppression, other data show no measurable effect on immune response at aflatoxin concentrations higher than commonly encountered in feedstuffs (154, 190, 192, 191, 246).

Pharmacological Interactions

Aflatoxicosis can influence drug effectiveness by altering the drug plasma half-life. Ceftiofur serum concentration was lowered (12). Chlortetracycline plasma concentrations were lowered due to decreased drug binding to plasma protein (358). Conclusions

differed on aflatoxicosis being spared or worsened by the addition of chlortetracycline to feed (318, 499).

Metabolism and Residues

Poultry reared on diets contaminated with aflatoxin constitute a minimal aflatoxin threat to the human food chain. Aflatoxins distributed to edible tissues in low concentrations and cleared rapidly after nontoxic diets were provided. In broilers, metabolites of aflatoxins B1 and B2 concentrated in gizzard, liver, and kidney (87) but cleared in 4 days. Aflatoxin B1 metabolized into conjugated aflatoxins B2a and M1 in the liver (97), and further reduced to aflatoxicol (418, 419). Aflatoxin B1 was excreted in the bile, urine, and feces as 6 major metabolites (222). In turkeys, aflatoxin B1, M1, and aflatoxicol concentrated in liver, kidney, gizzard, and feces and rapidly cleared (205, 458). Selenium supplementation increased the percentage of conjugated aflatoxin and partially protected turkeys (70, 206).

The half-life of aflatoxin B1 in laying hens is about 67 hours (473), and the feed:egg transmission is about 5000:1 (392). Most aflatoxin excreted through the bile and intestine, but aflatoxin B1 and aflatoxicol were detected in ova and eggs for 7 days or longer (268, 528). Aflatoxin B1 accumulated in reproductive organs with transfer to eggs (both yolk and albumen) and hatched progeny (yolk sac and liver) in chickens, turkeys, and ducks (503, 582).

Ochratoxins

Etiology and Toxicology

Ochratoxins are among the most toxic mycotoxins to poultry. *Penicillium viridicatum* and *Aspergillus ochraceus* produce nephrotoxic ochratoxins on grains and feedstuffs throughout North America, Europe, and Asia (reviewed 152). Ochratoxins are isocoumarin compounds linked to L-b-phenylalanine and are designated A, B, C, and D, and their methyl and ethyl esters. Ochratoxin A (OA) is the most common and most toxic, and is relatively stable. Some ochratoxin-producing fungi produce other mycotoxins toxic to poultry, including citrinin. Ochratoxin is the major determinant in porcine endemic nephropathy, a chronic wasting disease that caused failure to thrive in bacon pigs in Denmark and Ireland (reviewed 296).

Ochratoxin A occurs in North America, Europe, and Asia in corn (489, 488), most small grains and in animal feeds (76, 224, 281, 529, 580). Ochratoxin A contaminated moldy grain in game bird feeders (386) and ingredients for chicken feed, including sorghum, peanuts, sunflower, rice bran, and millet (524), some co-contaminated with aflatoxin.

Ochratoxin A readily forms in poultry feed under conditions of high temperature and high moisture (27). Ochratoxin A was the predominant toxin in spontaneous disease, with ochratoxins B and C detected only with high concentrations of OA (219).

Natural Disease

Ochratoxin in pelleted feed colonized with *A. ochraceus* and *Penicillium* spp. caused mortality and failure to gain weight in broilers (5). At necropsy, lesions were pale discoloration of liver and kidney, and enteritis. Contaminated corn or corn gluten meal caused ochratoxicosis in broilers (219). Renal disease affected growth, feed conversion, and pigmentation, and chickens developed airsacculitis. Ochratoxin and aflatoxin were linked to fragile intestines that tore and contaminated carcasses with intestinal content at processing (540).

Slaughter inspection identified poultry with enlarged pale kidneys (164). Kidney had OA residues, atrophy and degeneration of proximal and distal tubules and interstitial fibrosis. Ochratoxin A residues were similarly linked to pale enlarged broiler kidneys at processing, but factors in addition to the mycotoxin were contributory (509).

Ochratoxin contaminated corn caused feed refusal and mortality due to nephrotoxicity and airsacculitis in turkeys (219). Histopathology confirmed nephrosis as renal edema and necrosis of proximal tubular epithelium.

Two episodes of ochratoxicosis in hens caused by contaminated corn occurred as nephropathy and reductions in egg production and shell quality (407). Chronic renal disease and diarrhea caused yellow stains on the eggshells, resulting in decreased market value. Experimental feeding of OA caused a diarrhea with high urate content and the eggshells had yellow stains.

Ochratoxin A occurred in moldy bread and flour (398), which are components of bakery by-product, a feed ingredient. Moldy bread contaminated with ochratoxins A and B caused enteritis in chickens (549). A hepatopathy in geese and broilers was associated with ochratoxin contamination of corn. Geese had hepatopathy, gout, and mild nephropathy, and broilers had severe hepatopathy. An additional unidentified toxin was likely involved, and ochratoxin was considered an indicator toxin (486).

Experimental Disease

Experimental OA mycotoxicosis causes primarily renal disease but also influences hepatic, immunologic, and hematopoietic functions and has significant interactions with other toxins and nutrients.

Pathology

Acute lethal OA mycotoxicosis in chickens caused pallor of the liver, pancreas, and kidney; swelling of the kidney; white urate deposits in the ureter; and visceral urate deposition (145, 179, 251, 253, 421). The main histologic lesion was acute tubular nephrosis with focal necrosis of tubular epithelium, proteinaceous and urate casts, and heterophilic inflammation. Some chicks developed cytoplasmic vacuolation and focal necrosis of hepatocytes, followed by fibrosis. Suppressed hematopoiesis occurred in the bone marrow, and lymphocyte depletion occurred in the spleen and bursa of Fabricius.

Subacute OA mycotoxicosis increased weight of liver and kidney and decreased weight of lymphoid organs in turkeys, ducklings, and chickens. Ringneck pheasants and Japanese quail were the most sensitive game birds (468, 469, 470). Lesions were pallor of the kidney and catarrhal content in the intestine (149).

The histologic lesions among these species were tubular dilatation and cast formation in the kidney (73, 149, 253, 341). Hyperplasia of tubular epithelium and interstitial inflammation accounted for the kidney enlargement. Thickening of glomerular

basement membranes was dose related. The liver had vacuolar change in hepatocytes, which was associated with increases in glycogen content of liver and skeletal muscle in chickens (149, 257, 554). In ducks, the hepatocyte vacuolation was due to lipid accumulation (73), and in Japanese quail, it occurred with bile duct proliferation (143). Ochratoxin was toxic to mitochondria in the proximal renal tubules and in hepatocytes (61, 73, 150, 523). Severe lymphocytic depletion occurred throughout the immune system. Ochratoxin caused intestinal fragility associated with decreases in collagen (553) and heterophilic inflammation in the intestinal lamina propria and muscular layers (150).

Broilers fed OA developed soft bones with an increased diameter of the tibia relative to body weight and decreased breaking strength (150, 255). The bone histopathology showed osteopenia with disturbed endochondral and intramembranous bone formation (147). Osteoid formation was defective, and osteoporosis developed. Changes in the diaphyseal cortices accounted for the reduced breaking strength.

Chronic ochratoxicosis in hens caused reduced renal function that correlated with mild histologic lesions of ongoing necrosis and regeneration of tubular epithelium (295, 428).

Clinical Pathology

Ochratoxin A caused microcytic anemia involving iron metabolism (30, 256) and leukopenia (24, 82, 84). Coagulation disorders and decreased clotting factors occurred at OA concentrations too low to affect growth (145, 438, 134). Serum biochemical changes reflected damage to the kidney and liver (84, 262, 297, 475), as well as skeletal muscle, pancreas, and bone (163); and renal function was reduced (253, 295, 515).

Nutrition and Digestion

Experimental ochratoxicosis confirmed field observations of feed refusal by turkeys and leghorn hens but not broilers (68, 69, 441). Ochratoxin A impaired utilization of dietary carotenoids for carcass pigmentation (252, 475). The toxicity of OA was enhanced by vanadium and tannic acid (297, 300, 298). Combined exposure to OA and aflatoxin caused a synergistic reduction of growth and feed performance of broilers (546).

Reproduction and Egg Production

Ochratoxicosis in leghorn pullets delayed and blocked sexual maturity (99). The reluctance of hens to eat feed contaminated with ochratoxin caused reductions in body weight, egg production, and egg weight (438, 441). Ochratoxin reduced egg size, interior quality, and shell specific gravity at concentrations too low to influence the number of eggs produced (525). Japanese quail breeders experienced reductions of fertility and hatchability due to early embryonic death (439). In chickens, hatchability was reduced by embryonic mortality due to embryonic gout, and progeny had reduced growth (99, 384). Ochratoxin A was teratogenic for chicken embryos (193).

Immunosuppression

The immune system is a primary target of OA, seen as generalized atrophy and lymphocytic depletion of all lymphoid organs (72, 149, 292, 433). Ochratoxin A impaired cell-mediated immunity in broilers and turkeys (151, 493) and humoral immunity in broilers (148, 162). Other indicators of OA immunosuppression were impaired phagocytic activity of chicken heterophils and macrophages (83, 433); impaired vaccination responses (160); and increased severity of concurrent coccidiosis (259), salmonellosis (221, 211), and colibacillosis (312). The converse also applies, in that the renal lesions were more severe in broilers with coccidiosis (511).

Metabolism and Residues

Dietary OA distributed chiefly to the kidney with lesser concentrations to liver and muscle (180) Ochratoxin A was rapidly eliminated from chickens with a half-life of about 4 hours. Liver and kidney are the tissues of choice to monitor for residues (48, 356), and residues occurred in the absence of renal lesions (295). Residues persisted for 4 days or less when toxic diets were replaced (200, 440). Ochratoxin A distributed to egg yolk and albumin (175), which accounted for reductions in hatchability. Concentrations of OA in eggs had low correlation to dose, and several studies detected no OA in eggs (295, 438). In Japanese quail, OA was distributed to liver, kidney, proventriculus, and ovary and excreted in bile and in urine (176).

Citrinin

Etiology and Toxicology

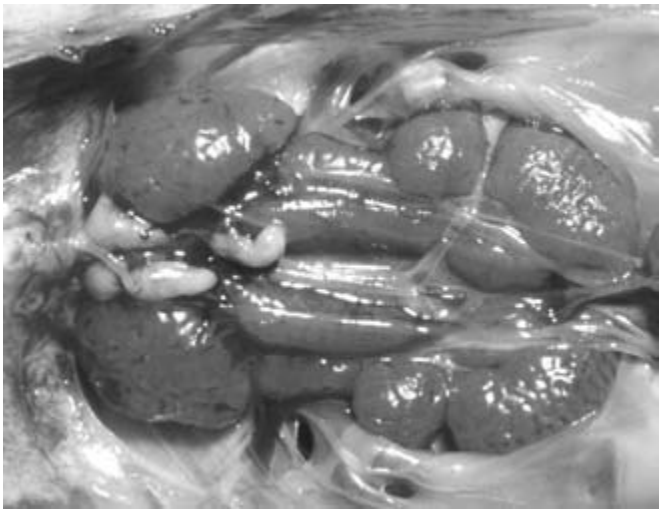
Penicillium (102) and *Aspergillus* (reviewed 455) produce citrinin, a natural contaminant of corn, rice, and other cereal grains. *Penicillium citrinum* occurs mainly in Canada and northern Europe (reviewed 580), suggesting toxigenic *Penicillium* may have a competitive advantage in cooler climates. First purified as a yellow crystalline compound from *P. citrinum* in 1931, recognition of antibacterial and antibiotic properties preceded the discovery of nephrotoxicity. Citrinin was one of the causes of yellow rice mycotoxicosis in Japan and linked to porcine endemic nephropathy, which also involved ochratoxin. Citrinin is heat sensitive.

Natural Disease

Penicillium lanosum, which produced citrinin in culture, was isolated from broiler feed in a house in which the litter was wet and chickens were substantially smaller than expected at slaughter (37, 377). Fungal cultures fed to broiler chicks caused watery fecal droppings and reduced weight gain. At necropsy, kidneys were swollen, and the gizzard lining was discolored and fissured. Histopathologic changes in kidney were swelling and pyknosis of tubular epithelial cells. *P. lanosum* was also isolated from mycotic lesions in the gizzard lining.

Experimental Disease

Citrinin is nephrotoxic and causes diuresis in poultry (199, 212, 341). Removal of the toxin allows a return to normal renal function. Citrinin acts directly on the kidney to increase urine flow rates and free water clearance with increases in sodium, potassium, and inorganic phosphate excretion (197, 239).



31.8. Experimental acute lethal citrinin mycotoxicosis in a chicken. The kidneys are swollen (352).

Pathology

A single dose of citrinin was nephrotoxic to chickens, turkey poults, and Peking ducklings, with turkeys being the most sensitive. Watery fecal droppings and increased water consumption accompanied swollen kidneys and histologic lesions of epithelial degeneration and necrosis in the proximal and distal tubules (352, 353) (Fig. 31.8). The liver had variable necrosis of hepatocytes, hemorrhage, and proliferation of bile ductules. Lymphocyte necrosis and depletion occurred in major lymphoid tissues, most prominent in ducklings (354, 351). Subacute to chronic toxicity in ducklings reduced weight gain and dose-related nephropathy with degeneration, necrosis, mineralization, and regeneration of tubular epithelial cells in cortical and medullary regions, interstitial inflammation, and fibrosis. Ultrastructural lesions in leghorn chicks showed that citrinin targeted epithelium in the proximal tubules (61).

Laying hens fed citrinin-appended diets developed wet droppings, but egg production and body weight were not affected (13).

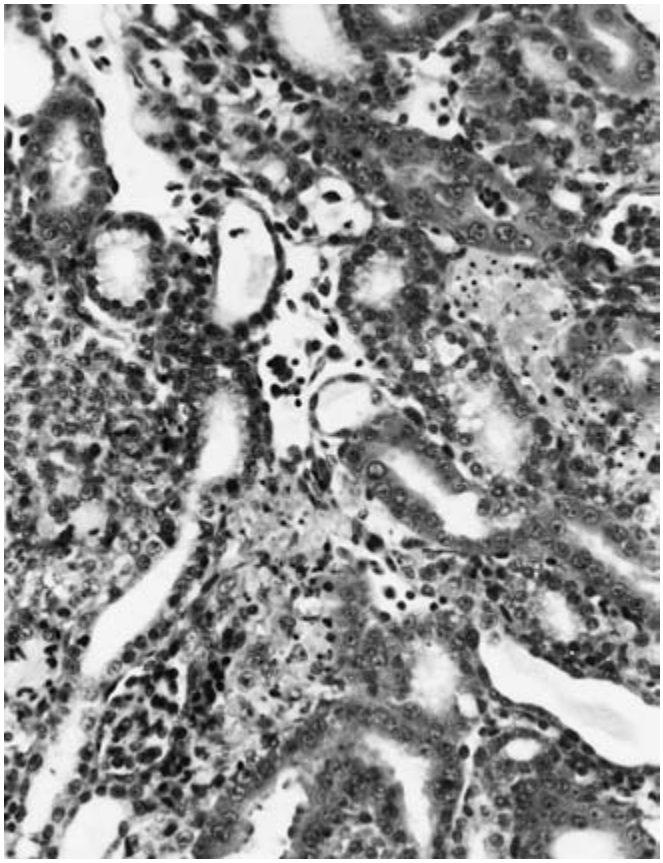
The histologic evidence of lymphocytic depletion is suggestive of potential immunosuppression; however, citrinin had no effect on humoral or cell-mediated immunity at nephrotoxic doses in broilers (75).

Clinical Pathology

Penicillium citrinum-contaminated corn containing citrinin caused anemia and leukopenia in leghorn chicks (462). Hyperkalemia, metabolic acidosis, reduced blood pH, and base excess occurred during intoxication (351).

Metabolism and Residues

Citrinin fed to broilers was detectable only in the blood and liver (378). Citrinin fed to laying hens for 6 weeks distributed to skeletal muscle, egg yolk, and egg white (1).



31.9. Kidney from a chicken fed oosporein for 3 days. The renal cortex has necrosis of proximal tubules (62).

Oosporein

Etiology and Toxicology

Oosporein is a red, toxic, dibenzoquinone metabolite of *Chaetomium* spp. and in poultry it causes high mortality from gout (107, 424). Oosporein originally was extracted from *Oospora colorans* (reviewed 578). *Chaetomium* spp. isolated from numerous feeds and grains, including peanuts, rice, and corn, are highly toxic in both plant and animal bioassays.

Natural Disease

Oosporein mycotoxicosis in both North and South America occurred as nephrotoxicity, gout, and mortality (578).

Experimental Disease

Pathology

Experimental oosporein mycotoxicosis in young chickens and turkeys caused visceral and periarticular urate deposition (gout) related to impaired renal function and elevated plasma uric acid (339, 340, 423, 424). Chickens fed oosporein for 3 days had necrosis of the proximal tubules in the renal cortex (62) (Fig. 31.9). Chickens were more sensitive to oosporein than turkeys. Water consumption increased and fecal droppings became fluid. Corn cultures of *Chaetomium*-producing oosporein were more

toxic to chickens than the purified organic acid of oosporein. Sodium and potassium salts of oosporein were more toxic than the organic acid, and their existence in cultures and naturally contaminated grains explained apparent enhanced toxicity (340).

Acute lethal doses of oosporein in chickens and turkeys caused dehydration, swollen pale kidneys, and extensive visceral gout (424, 425, 576). The liver was mottled and had focal necrosis, and the gallbladder was distended with bile. The proventriculus had an enlarged circumference; the mucosa was covered with exudate; and necrosis occurred at the isthmus. The gizzard lining and intestinal contents were discolored green.

Visceral gout is less pronounced or absent in subacute intoxication, but periarticular gout (white uric acid deposits in the joints) is apparent. Histologic lesions in the kidney occurred as proximal tubular nephrosis with periodic acid Schiff-positive granules in the macula densa (60). Interstitial granulomas around urate deposits in the kidney were common. Chicks that survived had renal lesions of interstitial fibrosis, hyperplasia of proximal tubular epithelium, and dilation of centrilobular distal tubules. Glomeruli were atrophic in fibrotic areas and enlarged in normal areas.

Oosporein reduced both feed consumption and egg production at doses capable of inducing nephrotoxicity and gout (576).

Clinical Pathology

Oosporein intoxication of broiler chickens had no hematological effect. Plasma uric acid concentrations were elevated in chickens and turkeys, and serum chemistry changes were generally reflective of renal toxicity (424, 425, 576).

Other Mycotoxins

Cyclopiazonic Acid

Cyclopiazonic acid (CPA) is a metabolite of *Aspergillus flavus*, the predominant producer of aflatoxin in feeds and grains. Some features of turkey "X" disease in the United Kingdom in 1959, notably enteritis and opisthotonus, were not fully accountable to aflatoxin and are explained by the presence of CPA, which was identified in stored samples from the original episodes (52, 108). Ten of 45 strains of *A. flavus* isolated from feed produced both CPA and aflatoxin in culture (347). The toxin is also produced by *Penicillium* spp. and is a contaminant of meats, outer portions of cheeses, peanuts, corn, and millet (315, 320).

In chickens, CPA affected weight gain and feed conversion, and caused mortality (141). It had additive toxicity with aflatoxin and T-2 toxin (305, 496). Lesions occurred in the proventriculus, gizzard, liver, and spleen. The proventriculus lumen was dilated, and the mucosa thickened by hyperplasia and ulceration (188). Mucosal necrosis occurred in the gizzard. Liver and spleen contained numerous yellow foci of necrosis and inflammation. Lymphocyte depletion from the thymus and spleen involved apoptosis (282, 545).

The reproductive tract of male broiler breeders was impaired (514).

Cyclopiazonic acid residues occurred in chicken muscles at 14% of an oral dose, 48 hours after dosing (385), and in eggs, with higher concentrations in albumen (142).

Sterigmatocystin

Sterigmatocystin, a biogenic precursor to aflatoxin B1, is hepatotoxic and hepatocarcinogenic. It occurs less commonly than aflatoxin and is associated with visibly moldy products (432). Sterigmatocystin is produced on small grains, coffee beans, and cheese by *Aspergillus versicolor* and other *Aspergillus* spp., *Chaetomium* spp., and other cereal fungi, with detection in North America, Europe, and Japan (reviewed 580). Sterigmatocystin is less toxic than aflatoxin but produced in higher concentrations (479).

Sterigmatocystin mycotoxicosis occurred in laying hens fed commercial crumbled feed colonized with *A. glaucus* and containing sterigmatocystin (5). Feed intake and egg production decreased, and brown-shelled eggs were pale. At necropsy, the livers were pale, fatty, and contained hemorrhages.

Experimental sterigmatocystin mycotoxicosis in Leghorn chicks affected liver, pancreas, lymphoid organs, and kidney (505, 506). Histologically, the liver had congestion and hemorrhage, and necrosis of periacinar hepatocytes accompanied by heterophils. Pancreas had cytoplasmic vacuolation from zymogen-granule loading in exocrine cells. Lymphocyte necrosis and depletion occurred in lymphoid organs. Kidney had mild degeneration and necrosis of tubular epithelium. Serum chemistries reflected target organ injury, and there was leukopenia.

Sterigmatocystin caused reduced embryonic weight, malformations, and mortality in chicken embryos (479). In combination with a low dose of aflatoxin, adverse changes occurred in poultry meat and in hematological parameters (2).

Rubratoxins

Rubratoxins A and B are hepatotoxic mycotoxins produced by *P. rubrum* and *P. purpurogenum* and were recognized before aflatoxicosis was defined (reviewed 560). In 1958, an investigation of poultry hemorrhagic syndrome yielded these fungi from feed and litter of affected chickens (172). It is noteworthy that *A. flavus* and *P. citrinum*, producers of aflatoxins and citrinin, respectively, were also studied (173). Chicks fed cultures of *P. rubrum* and *P. purpurogenum* developed bloody diarrhea. At necropsy, hemorrhages occurred in muscles and viscera, and erosions and free blood were in the proventriculus and gizzard.

Purified rubratoxin (20% A, 80% B) however showed relatively low toxicity for chickens (567). Acute lethal intoxication produced congestion and hemorrhages. Dietary rubratoxin impaired growth and caused liver enlargement and atrophy of the bursa of Fabricius. Hemoglobin, serum protein, and serum cholesterol decreased and capillary fragility increased.

Penicillic Acid

Penicillic acid, discovered in 1913, is a metabolite of numerous species of *Penicillium* and *Aspergillus* (reviewed 258) and is potentially important to poultry because of high concentrations in corn and poultry feed. Penicillic acid has low toxicity for broilers, and purified toxin had minimal effect when fed solely at concentrations likely to occur naturally. Growth and feed conversion were affected however when fed with low doses of aflatoxin (2).

Tenuazonic Acid and Alternaria *Toxins*

Tenuazonic acid, a metabolite of *Alternaria* spp., has a spectrum of biological effects (reviewed 189). Investigations of poultry hemorrhagic syndrome revealed marked toxicity by an isolate of *Alternaria* (173). *Alternaria* spp. grow on corn and other commodities and also produce alternariol, alternariol methyl ether, and altertoxin (reviewed 580). Tenuazonic acid had moderate toxicity for broilers and Leghorns. Acute lethal intoxication induced hemorrhages in skeletal muscle, heart, and subcutis. Subacute intoxication also caused hemorrhages, and erosions occurred in the gizzard lining.

Tenuazonic acid-producing cultures of *Alternaria longipes* were highly toxic to chicks and caused hemorrhages in the proventriculus and erosion of the gizzard lining (144, 500).

Patulin

Patulin is a mycotoxin produced by several species of *Aspergillus*, *Penicillium*, and *Byssochlamys*. Patulin-producing *Penicillium* spp. were isolated from chick starter feed (102, 333). Patulin had relatively low toxicity for chicks but produced sequential lesions of watery crop content, acute ascites, and hemorrhage in the lumen of the proventriculus, gizzard, and intestine. Growth was suppressed when fed in combination with low doses of aflatoxin (2). Hens fed patulin produced misshapen eggs with reduced calcium content in the shell (1).

Other Mycotoxins and Toxigenic Fungi

Kojic acid was mildly toxic to liver and kidney, and caused anemia in broilers but only at concentrations higher than would be likely encountered in feed and there were no significant interactions with aflatoxin (194, 195). Tremorigens, slaframine, and other toxigenic fungi examined for toxicity in poultry, including *Diplodia maydis*, *Phomopsis leptostromiformis*, *Helminthosporium maydis* Race T, and uncharacterized metabolites of *Penicillium citrinum*, were reviewed in a previous edition (247).

Diagnosis

Diagnosis of a mycotoxicosis begins with the assessment of the clinical history and signs. The onset of a problem may coincide with the delivery of new feed. Contaminated equipment for transportation, milling, and feeding can be the source of intermittent or chronic toxin exposure. Clinical signs and lesions caused by mycotoxins are not pathognomonic. For example, oral lesions are cited as evidence of trichothecene mycotoxins (T2-toxin), but similar lesions are caused by high concentrations of fines (small particles) in the feed (118, 370), copper sulfate, quaternary ammonia disinfectants, candidiasis, and hypovitaminosis A.

A definitive diagnosis of mycotoxicosis involves identification and quantification of specific toxins. This is difficult in modern poultry production because of the rapid and high volume use of feed and ingredients. The rather limited analytic capability of diagnostic laboratories (403) and the biochemical complexity of finished feeds are additional factors. Analyses for aflatoxin and zearalenone are readily available, but analyses for ochratoxins, zearalenol, DON, T-2 toxin, DAS, ergot alkaloids, and citrinin are less so. Confirmation of other trichothecenes, cyclopiazonic acid, sterigmatocystin, rubratoxin, or less common mycotoxins, especially in feeds, is possible in relatively fewer laboratories.

Analytic techniques for mycotoxins include chromatography (thin-layer, gas, liquid), mass spectrometry, and monoclonal antibody-based technology that use enzyme-linked immunosorbent assay (ELISA) technology. ELISA test kits should be used for validated applications as interfering substances in ingredients and feeds may give inaccurate results. Some assays are standardized and validated for grain and certain other ingredients but not for finished feeds, which can be a variable substrate for extraction and analysis. The black light evaluation of grains for *A. flavus* growth is an acceptable presumptive test for aflatoxin but does not confirm actual toxin (51). Numerous bioassays are defined for mycotoxin screening tests, but positive test results are presumptive.

Laboratory personnel should be consulted before sending samples, as laboratories differ in the capabilities to conduct screening and confirmation tests for mycotoxins. Identification of mycotoxin residues in blood or tissues is possible but not routinely available (238). An ELISA test for confirmation of aflatoxin in liver showed high sensitivity and specificity and would complement diagnostic investigation (186).

If a mycotoxicosis is suspected, a complete diagnostic evaluation is desirable in addition to feed analysis. A flock rarely experiences a single disease stress and other diseases may be occurring in concert with mycotoxins to affect production adversely. A suspected mycotoxicosis may not be confirmed by feed analysis; however, a complete laboratory evaluation can exclude other significant diseases (248). Birds that recently died and those obviously sick should be selected for submission.

Moldy feed appears unwholesome and indicates the potential for mycotoxin formation. It can be unpalatable and have reduced nutritive value, with vitamins, amino acids and the energy level of fats affected (68, 34, 35, 366, 461). *Aspergillus*, *Penicillium*, and *Fusarium* are mycotoxin-producing fungal genera that occur in most poultry feeds, so the potential is clearly evident (368).

Feed and ingredient samples should be properly collected and promptly submitted to a feed testing laboratory for analysis. Mycotoxin formation may not be uniform in a batch of feed or grain, and multiple samples from different sites, including areas with caked or moldy feed, increase the likelihood of confirming a mycotoxin formation zone (hot spot). Sampling is usually the largest source of variability associated with a mycotoxin test procedure (558). Samples should be collected all along the chain of ingredient storage, feed manufacture and transport, feed bins, and feeders within poultry houses. Fungal activity increased as feed moved from the feed mill to feeder pans (277) and was associated with an increase in fines and higher zinc concentrations.

Samples of 500 g (1 lb) should be collected and submitted in separate containers. Clean paper bags, properly labeled, are adequate. Sealed plastic or glass containers are appropriate only for short-term storage and transport because grain rapidly deteriorates in airtight containers. A written record of sample collection and the application of labels directly to specimen containers (not the lids) help to ensure sample integrity.

Rapid on-site screening tests for several mycotoxins (aflatoxin, T-2 toxin, DON, fumonisin, ochratoxin, and zearalenone) are available in monoclonal antibody-based detection kits. Grain can be screened for potential aflatoxin contamination by examination for green fluorescence under a black (ultraviolet) light to estimate the degree of *A. flavus* contamination. The presence of aflatoxin, however, must be confirmed by a chromatographic procedure (minicolumn technique) or other suitable method.

Treatment

Toxic feed should be removed and replaced with unadulterated feed. Poultry generally recover from most mycotoxicoses soon after an uncontaminated diet is available. Treatment of ongoing parasitic or bacterial diseases will alleviate additive or synergistic interactions. Substandard management practices are especially detrimental to poultry stressed by mycotoxins and should be corrected. Vitamins, trace minerals (especially selenium), protein, and lipid requirements are increased by some mycotoxins and can be compensated by feed formulation and water-based treatment. Increasing the crude protein, dietary energy, and vitamin supplementation can counteract the effects of aflatoxin (3, 46, 277). Dietary amendment with either sunflower or soybean oil alleviated some effects of aflatoxin (450). Vitamins E and C partially counteracted the toxicity of T-2 toxin and ochratoxin A in chicks (3, 240, 269).

Another strategy for treatment of mycotoxicoses in the presence of contaminated feeds involves specific nutrients that metabolize to form detoxicants (375, 431). Supplemental methionine and N-acetylcysteine counteracted aflatoxin likely through the enhancement of glutathione formation (128, 294, 542).

Prevention

Although overt intoxication by mycotoxins is relatively uncommon, contamination of feedstuffs by fungi capable of producing mycotoxins is quite common. Many mycotoxins described in this chapter have subclinical effects on hatchability, shell quality, immunosuppression, and processing parameters, at toxin concentrations too low to register as diagnostically significant. Preventing the insidious effects of mycotoxins by an integrated program to prevent mycotoxin formation and inhibit the effects of mycotoxins already formed can substantially benefit poultry health and production.

Feed Manufacturing and Management

Prevention of mycotoxicoses centers on using mycotoxin-free feedstuffs and feed manufacturing and management practices that prevent mold growth and mycotoxin formation. This ideally involves access to analytical capability to confirm the purchase of ingredients free of mycotoxins, or at least, the type and degree of contamination so that risk can be assessed and addressed. Proper storage of ingredients, and feed processing, shipping, and handling procedures are necessary to minimize mycotoxin formation. A quality control program can monitor success of these practices (521).

Manufacturing and maintaining feed of low moisture (11–12%), keeping the feed fresh, and maintaining clean feed handling equipment are key control points in preventing mold growth (382). Mycotoxins form in decayed, crusted, built-up feed in feeders, feed mills, and storage bins (218). Regular inspection of feed bins identifies flow problems like feed separation, central feed-down, and feed bridging (526), which enhance fungal activity and mycotoxin formation. Temperature extremes cause moisture condensation and migration in bins and create high-risk situations for mycotoxin formation (577). Bin inspection and cleaning between flocks to certify absence of feed residue are a practical control point. Tandem feed bins on farms allow cleaning between successive feed deliveries. Minimizing feed residence time on the farm is important, even under cool, dry conditions (202). Adequate ventilation of poultry housing to reduce relative humidity removes moisture available for fungal growth and toxin formation in feeders (276). Selection of feeder equipment that minimizes surface-area contact with feed diminishes mycotoxin formation (278).

Pelleting feed destroys some fungal spores (520) and generally decreases the fungal burden. Pelleting is made further effective by the addition of an antifungal agent.

Antifungal Agents

Antifungal agents added to feeds to prevent fungal growth have no affect on toxin already formed but may be effective with other feed management practices. Regulatory approval of these various compounds in feeds differs among countries. Organic acids are effective against *Fusarium*, *Aspergillus*, and *Penicillium*, in order of declining susceptibility (132, 235, 280). The effectiveness of organic acids may be influenced by ingredient particle size and buffering by certain ingredients (133, 131). Organic acids are generally corrosive and irritating to skin, but some have been modified to counteract this (417). Other agents showing efficacy in reducing fungal growth or mycotoxin formation include phosphates (tetrasodium pyrophosphate and alkaline sodium polyphosphate) (321), spice oils and extracts (146, 227, 502), and ammonium hydroxide (187). Gentian violet (88, 289) and thiabendazole (177) are effective but no longer approved for use in the United States for animal feeds. Copper sulfate is a poor mold inhibitor for poultry feeds (120). Other antifungal strategies include controlled storage atmospheres, irradiation, and fumigation (328).

Detoxification with Binding Agents

Detoxification using mycotoxin-binders holds promise for using contaminated feeds while preventing intoxication (reviewed 431). Inorganic mineral adsorbents or binders including various clays, soils, and zeolites can be part of an integrated approach (302, 335, 336, 388, 389, 390, 395). Zeolites are silica-containing compounds that are practical and economical feed additives that can reduce the effects of aflatoxin and cyclopiazonic acid (32, 153, 225, 323) but not T-2 toxin, DAS, or ochratoxin A (263, 303, 310, 472, 183). The adsorbent activity is shown by a number of zeolitic ores (225, 346, 395, 397) and bentonite clay (284, 355, 550). Further processing of silicate-type binders may

increase their efficacy for protection (304). Adsorbents can counteract mycotoxins but vary in efficacy and in the correlation of *in vitro* and *in vivo* testing (64)

Polyvinylpolypyrrolidone, a synthetic binder, diminished the toxicity of aflatoxin for chickens (78, 290).

Progress continues in the development and application of organic detoxification compounds. Esterified glucomannan, a cell wall derivative of yeast used in beer fermentation, *Saccharomyces cerevisiae*, was protective against aflatoxin and ochratoxin in broilers (449, 507, 283) and showed moderate binding activity for fumonisins, zearalenone, and T-2 toxin (128). Esterified glucomannan is generally protective against naturally contaminated feed containing multiple toxins (18, 449, 516). Some products are combinations of enzyme detoxicants and binders, and nutrient supplements (123, 130). Other organic natural compounds are under investigation and show some potential (449, 510, 512).

Other methods of prevention include ozone treatment of grain, which reduced aflatoxin contamination (348). Ammoniation was effective in decontaminating feeds and grains for aflatoxin (413), and composting contaminated corn with poultry litter was effective in detoxifying aflatoxin (279). Certain cultures of *Lactobaccillus* prevented aflatoxin absorption from the small intestine (161). Superactivated charcoal (fine particle size) had marginal or no binding activity for aflatoxin or T-2 toxin (158). Long-term efforts to prevent mycotoxin exposure involve developing grain-producing plants that resist fungal infection, reduce the toxic effects of the mycotoxins, and interrupt mycotoxin biosynthesis (104).

Public Health Significance

The history of mycotoxicoses contains many accounts of food poisonings, including ergotism, alimentary toxic aleukia and related syndromes caused by trichothecenes, and yellow rice poisoning (reviewed 38). Most grains used as feedstuffs for poultry and livestock are also ingredients for human food and carry the same risks for fungal growth and mycotoxin contamination. In addition, foods such as coffee, tea, spices, and dried fruits support fungal growth and toxin formation. Surveys of foodstuffs are ongoing worldwide and document toxin contamination at moderately high prevalence but usually at low concentrations. Coffee (331), wheat, cereal grains, and flours (165, 478), spices (406), and rice (415) are typical foods from which ochratoxin and trichothecenes and other fusarial toxins can be detected. Peanuts are closely monitored for aflatoxin and cyclopiazonic acid (371, 513).

Poultry products present low risk for human toxin exposure because mycotoxins have minimal distribution to skeletal muscle, the major form of poultry consumption. Mycotoxin distribution to liver, kidney, and eggs is of greater concern. Mycotoxins are rapidly metabolized and excreted in urine or feces. Residues in liver and kidney are generally transient and in low concentrations relative to the exposure dose. Chronic exposure to mycotoxins causes changes in color and size of liver and kidney, a visible indicator for rejection from the food chain at slaughter. In modern production, mycotoxin exposure of poultry is somewhat self-limiting because the adverse economic effects make prevention of exposure important.

In the United States, the Food and Drug Administration (FDA) has an action level of 20 parts per billion (ppb) for aflatoxin in foods (which would include poultry and eggs) and 0.5 ppb for aflatoxin M1 in milk (15). The FDA has a guidance level of 2 to 4 parts per million for fumonisins for corn products intended for food (16). Because of the global nature of food production and international movement of grains and food commodities, international standards and surveillance are important. Broadening the safety net to prevent mycotoxin exposure in the food chain requires international cooperation for safety, risk assessment, action levels, and advisories (414).

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Chapter 32

Other Toxins and Poisons

Richard M. Fulton

Introduction

Paracelsus recognized more than 400 years ago that it is "the dose that makes the poison." Although that may be obvious with known toxic materials, it is also true for more benign products such as growth promotants and chemotherapeutic agents usually considered safe. Deliberate or inadvertent overdosages may cause illness, and a misplaced decimal in water or feed medication frequently results in toxicity. A general feature of modern complex poultry rations, feed mill equipment, and feed delivery to poultry farms is that any component included in a ration will at some time be mistakenly included at a higher than desired rate. This may occur through human or mechanical error. Furthermore, when toxicoses do occur, they are added to the preexisting background of complicating infectious agents, vaccine administrations, and environmental exposures. This usually leads to multi-factoral mixed clinical presentations of toxic, infectious, environmental, and management diseases, rather than "pure" toxicoses. Additionally, some primary highly pathogenic infectious or environmental diseases of poultry will present such rapidly elevating mortalities that acute toxicoses are suspected incorrectly.

For example, sulfaquinoxaline poisoning occurs in meat-type chickens, even at recommended doses, because of high water intake in warm buildings, particularly in hot weather, or because of poor feed mixing. Disease may also be caused by toxic levels of some nutrients (e.g., excess dietary sodium causes significant losses in chickens and turkeys around the world). High levels of vitamins A and D are toxic. Some compounds have differential species or age toxicities, and others have increased toxicity in naive animals with no previous adaptative exposure. Ionophore anticoccidials commonly exhibit such differential adaptive resistance to toxicity. Furthermore, waterfowl are sensitive to some drugs at a dose safe for chickens and turkeys. The immune system seems to be affected by many toxic agents. In addition to disease caused by poisons, the problem of residues in eggs and meat must also be considered. For information on drugs approved in the United States, withdrawal times, and drug and chemical residues, see Booth (31), www.fda.gov/cvm/greenbook.html, www.compasnac.com, or the current Feed Additive Compendium. In rationalizing withdrawal times in egg-laying chickens, it must be remembered that compounds begin to be deposited in chicken egg yolk 10 days before that egg is laid.

Poisonous substances are widely distributed in nature. Mycotoxins, covered in the previous subchapter, are important to the poultry industry, but toxic agents are also produced by bacteria (botulinum toxin, methylmercury, toxic amines) or occur naturally (selenium, phytotoxins). Pesticides, herbicides, and other synthetic chemicals, metals such as lead, and industrial contaminants add to the list of toxic materials. Many chemicals and human drugs have been given to birds in feed and water to study their toxic effects. These experimental toxicities generally have not been included in this chapter, except as they may relate to potential naturally occurring or iatrogenic poisonings in poultry.

Poisons and toxins are not major causes of production loss or disease in poultry in most countries, although some, such as lead, pesticides, and botulism, are significant in wild birds. In 1985, Terzic and Curcic (318) reported, however, that 40% of 2065 poisoning cases seen at the Belgrade Veterinary Facility during a 17-year period were in poultry. In 2005, Sharpe and Livesey (288) reported that 1.4 % of 876 poisoning cases of food safety concern that were seen at the Veterinary Laboratories Agency in England and Wales were in poultry, most of which were lead poisoning in waterfowl. Poisoning occurs more frequently in free-range and backyard flocks and in village poultry where birds forage in neighboring gardens and fields or receive household waste and weeds cut from roadsides and fields. Some of these poisonings are malicious. Contaminated litter on the floor and in nest boxes is an added source of toxins in chickens not raised on wire. Because suspected toxicity cases are more likely to be submitted to a diagnostic laboratory than are other sick birds, statistics collected from that source may not be an accurate indication of the incidence of poisoning compared with other disease.

Toxicants covered in this chapter are presented by primary use. Levels of toxic substances that may cause depressed growth in broilers and turkeys or decreased egg production in layers are summarized in Table 32.1.

Antimicrobials, Anticoccidials, and Growth Promotants

Most reports of poisoning with chemotherapeutic agents involve inappropriate use or overdose of ionophore anticoccidials or growth promotants. Toxicity of a variety of chemotherapeutic agents in poultry and pigeons has been reviewed (266, 267).

Sulfonamides

Sulfonamides were used as the primary form of prevention and treatment for coccidiosis in poultry between the early 1940s and late 1950s. Sulfaquinoxaline and sulfamethazine were most

Table 32.1. Levels in feed (unless otherwise noted) of selected toxins documented to decrease growth rate in broilers and turkeys and reduce egg production in layers.

Toxin	Broilers	Turkeys	Layers
Antimicrobials and Growth Promotants			
Sulfadimethoxine (% in water)	NA[a]	NA	0.05
Sulfaquinoxaline (%)	NA	NA	0.10
Nicabazine (mg/kg)	NA	NA	70
Arsanilic acid (mg/kg)	1000	400	NA
Nitarsone (mg/kg)	300	600	NA
Roxarsone (mg/kg)	90	550	NA
Nutrients and Other Feed- and Water-Related Toxicants			
Aluminum (%)	0.30	0.30	0.15
Arsenic (inorganic pentoxide) (mg/kg)	40	40	40
Boron (mg/kg)	435	435	870
Boric acid (mg/kg)	2500	2500	5000
Cadmium (mg/kg)	400	400	8-60
Copper (mg/kg)	500-1000	500-1000	1000
Fluoride (mg/kg)	1300	1300	1300
Iodine (mg/kg)	500	500	300
Iron (mg/kg)	200-2000	200-2000	NA
Lead (acetate) (mg/kg)	630	630	630
Mercury (mg/kg)	50	50	5
Molybdenum (mg/kg)	200	200	200
Potassium (%)	0.90	0.90	NA
Selenium (mg/kg)	5	5	80
Sodium (%)	0.80	0.80	0.80
Sodium chloride (%)	2.0	2.0	2.0
Tungsten (mg/kg)	1000	1000	1000
Vanadium (mg/kg)	6	6	20-30
Zinc (mg/kg)	NA	NA	20,000
Other			
Ammonia (ppm)	**50**	**25**	**75**

[a]NA—not available.
Source: 77, 235, 236

widely used. The toxic level of sulfonamides is close to the therapeutic level in poultry, and even the therapeutic level has a detrimental effect on hemopoietic and immune systems. Previous low-level or continuous-preventive medication has a protective effect against subsequent higher doses (96).

Sulfonamides are difficult to mix evenly in feed, and they have low solubility in acidic water. These characteristics may cause some birds to receive a toxic dose even when appropriate treatment levels are added to bulk rations or water supplies. This is less likely at lower preventive levels. Both feed and water medication require accurate estimates of daily consumption if each chicken is to receive a therapeutic and nontoxic daily dose. Sulfa poisoning has occurred when no allowance was made for increased water and feed consumption of the modern broiler that eats to its physical capacity rather than to its metabolic need, or, more frequently, for the effect of increased water consumption at high environmental temperatures or in hot broiler houses. For broilers, previous authors of this chapter recommended one-half of the therapeutic dose, and at temperatures greater than 27°C (81°F), one-third of the therapeutic dose for water medication. Repeat treatment is hazardous and should not be recommended without a postmortem examination of a subset of individuals to make sure that there is no evidence of sulfa toxicity. Even the newer so-called safe sulfas need to be used with care (61, 267). Under no circumstances should sulfas be given simultaneously in both the feed and water. Decreased solubility in acidic water may lead to delayed clearance of sulfas from water lines and result in detectable drug levels in meat and eggs beyond published withdrawal times.

Hemorrhagic syndrome, which occurred frequently when sulfas were in widespread use, is a manifestation of sulfa toxicity and occurs at and above therapeutic dose levels. In addition to blood dyscrasia, bone marrow depression, and thrombocytopenia, sulfonamides depress the lymphoid system and immune function in birds. Similar but more dramatic hematologic manifestations and diatheses are seen in domestic mammals given sulfa-containing poultry rations or water medications. Focal bacterial granulomas are often found in tissues and organs of chickens dying from sulfa poisoning. Epithelial necrosis and degeneration in the liver, kidney, and other organs may be caused by the direct effects of the drug, or hypoxia secondary to drug-induced

anemia. When determining withdrawal times in chickens whose eggs reach the human food chain, deposition in the yolk 10 days prior to the production of an egg must be considered (29).

Signs

Chickens and turkeys with sulfa toxicity are depressed, pale, and frequently underweight. In adults, there is a marked decrease in egg production and shell quality; brown eggs may be depigmented (68, 242). Secondary bacterial infections including septicemia and gangrenous dermatitis may follow sulfonamide toxicity (61).

Pathology

For descriptions of gross and microscopic pathology, see references 61, 68, and 97.

Hemorrhage in skin, muscles, and internal organs is the most consistent and extensive gross lesion of sulfonamide intoxication. Hemorrhage may be present in comb, eyelids, face, wattles, anterior chamber of the eye, and musculature of breast and thighs. Normal dark-red bone marrow in growing birds changes to pink in mild cases and yellow in severe cases. The entire length of the intestinal tract may be spotted with petechial and ecchymotic hemorrhages, and the cecal lumen may contain blood. Hemorrhage may be present in the proventriculus and beneath the ventriculus (gizzard) lining. There may be ulcers at the proventricular-gizzard junction. The liver is swollen, pale red, or icteric and may be studded with petechiae or foci of necrosis. The spleen is commonly enlarged, has hemorrhagic infarcts, and contains gray nodular areas. "Paintbrush" ecchymotic hemorrhages occur in the myocardium. Thymus and bursa of Fabricius are small.

Microscopically, areas of caseous necrosis surrounded by a mantle of giant cells occur in liver, spleen, lungs, and kidneys. Lymphocyte and heterophil infiltrates are present at the periphery of necrotic foci. Lymphoid hypoplasia around splenic sheaths, edema and fibroplasia of the capsule, and macrophages containing hemosiderin are common. Early changes in the liver are periportal mononuclear infiltration associated with bile duct hyperplasia. Hemosiderin deposits are present in necrotic areas, and thrombosis of portal vessels is present. An early change in kidneys is interstitial lymphocytic infiltrate, but this may be associated with concomitant infections. Degeneration and necrosis of tubular epithelium are associated with hyaline casts. Glomeruli are enlarged, and Bowman's capsule is dilated with hyaline material. Lungs are congested with interlobular and interstitial edema. Interstitial tissues contain mononuclear foci. There is degeneration and necrosis of lymphocytes and depletion of bursal follicles.

In femoral bone marrow, there is decreased intrasinusoidal erythropoiesis with thrombocytopenia and agranulocytosis, focal increase in extrasinusoidal lymphopoiesis, and, in some instances, myelopoiesis. There are also focal areas of hyalinization, necrosis, and fibroplasia. Hemosiderin deposits and extrasinusoidal edema are present.

Nitrofurans

Nitrofuran use is no longer permitted in some countries. Prominent signs of nitrofurazone (NFZ) toxicity in chicks include depression, incoordination, ruffled feathers, and growth retardation (242). Poor growth may be partially related to feed aversion, because feed consumption drops as the level of NFZ increases. Ducklings on toxic levels of NFZ die suddenly without clinical signs. Nervous signs and hyperexcitability have been described in acute toxicity in chicks and poults. Loud vocalization, opisthotonos, aimless running and flying, and convulsions may be seen. Acute nervous signs of NFZ toxicosis disappear within hours after supplying unmedicated feed and water. Chronic exposure to furazolidone also may reversibly delay sexual maturity in male broiler breeder chickens (10, 299).

Furazolidone (FZ) toxicity mainly affects the heart in turkeys, ducks, and chickens (58, 213). Marked individual and age susceptibility to FZ occurs. Some poults, chicks, and ducklings grow well without cardiac damage when fed 400–700 mg FZ/kg feed; whereas others fail to grow, develop ascites, and signs associated with heart failure. Frequency and severity of clinical signs are dose related. Recovery occurred in affected ducklings (334). There also is clinical evidence that FZ may cause nervous signs in chicks and poults and infertility in male breeders (266).

FZ causes dose-related biventricular cardiomyopathy with prominent dilation of ventricles and thinning of either the right or left ventricular wall. Secondary heart failure results in passive congestion with lung edema, or marked congestion of liver and other organs, and ascites depending on whether heart failure is mainly left- or right-sided. Right-sided heart failure with marked cardiac enlargement is usually more common up to 3 weeks of age (167).

In turkeys, FZ-induced cardiomyopathy cannot be distinguished from spontaneous turkey cardiomyopathy (STC). The cause of STC is not known, but clinically it is associated with rapid growth, low serum protein, and stressors such as low incubator oxygen, poor ventilation, and fumes from brooders, which might induce ischemic cardiomyopathy. The mechanism of how high doses of FZ cause dilatory cardiomyopathy is not known (127).

Most microscopic lesions result from heart failure. Cardiac lesions include edema, thinning of myocardial muscle fibers, and multifocal myocytolysis with increased connective tissue. Epicardial fibrosis and endocardial fibroelastosis may also occur. Ultrastructural changes include myofibrillar lysis, clumps of Z-band material, and increased glycogen in myocardial fibers. Changes in heart muscle enzyme levels accompany tissue alterations.

Aminoglycoside Antibiotics

After subcutaneous injection, gentamicin (an aminogylcoside) causes depression in turkey poults, edema and hemorrhages at the injection site, and large, pale, and nephrotic kidneys (25, 276). Aminoglycosides and various other antibiotics used for egg inoculation have caused embryo mortality. Streptomycin and dihydrostreptomycin sulfate injected intramuscularly for sinusitis in turkey poults causes respiratory distress, paresis, and mild convulsions (266, 267).

Ionophore Antibiotics

Ionophores (ion carriers) facilitate movement of some monovalent cations, such as sodium and potassium, and divalent cations

such as calcium and magnesium across cell membranes. They can have both anticoccidial and antibacterial activity, and the group is used extensively in poultry and ruminant feeds. Ionophores are coccidiocidal because of their ability to preferentially move ions, usually Na+, into various stages of the parasite.

Toxic levels of ionophores cause potassium to leave and calcium to enter cells, particularly myocytes, resulting in cell death. Signs of toxicity are related to high extracellular potassium and high intracellular (intramitochondrial) calcium. For more specific information on metabolism and toxicity of monensin, see references 31, 81, and 234. Ionophore toxicity varies with species and age; equidae are very susceptible, and adult poultry, particularly turkeys, are more susceptible than broilers (130, 151, 288). There is a synergistic effect with antibiotics in the same family of drugs (323) and increased toxicity with nonrelated antibiotics, other drugs (33, 39, 80, 185, 246, 252, 266), and low-protein rations (267). Dehydration because of diarrhea or periods of water and/or feed deprivation can precipitate toxic events (46, 124). Monensin, lasalocid, salinomycin, and narasin have been associated with toxicity in poultry, guinea fowl, quail, and other species (64, 124, 132, 204, 255, 283, 288, 336). Lethal toxicoses have been described in equidae, and other mammals accidentally exposed to ionophore-containing poultry rations. Poultry have an adaptative resistance to dietary ionophores, and an inverse age sensitivity, with adult naïve poultry being more susceptible than young previously exposed birds.

Signs

Signs vary from anorexia with depression, weakness, and reluctance to move to complete paralysis in which birds lie in sternal recumbency with neck and legs extended. Less severely affected birds may show posterior paralysis with legs extended. Dyspnea has occurred in affected adult turkeys (Fig. 32.1). Signs are associated with muscle damage. Death may follow respiratory failure or be secondary to dehydration. Mortality is variable but may exceed 70% (102). In some cases of suspected ionophore toxicity in turkeys, morbidity may be low with only a few poults paralyzed. The term *knockdown syndrome* has been used for this condition (46). Poults with botulism may show similar signs. Reduced egg production (336) and fertility with weak chicks also have occurred (247).

Pathology

Subchronic monensin toxicity (329) resulted in opaque fibrin plaques on the epicardium, hemorrhage in coronary fat, and decreased liver weight. In acutely affected turkeys, pallor and atrophy of mainly type I fibers of legs and back have been observed associated with monensin use (24, 250, 328). However, clinical signs and gross lesions are often absent in breeders ingesting high levels of monensin (102).

Microscopic changes in heart and skeletal muscle consist of scattered areas of hyalinization with muscle necrosis and myofiber degeneration and necrosis. Birds with respiratory signs often have lesions in tracheal muscles. Type I fibers appear to be selectively affected (132). Heterophils, macrophages, and occasionally lymphocytes may be present. Frequently, when exposed to low doses or interaction with other drugs occurs, affected areas are very cellular with large numbers of satellite or sarcolemmal nuclei, indicating regeneration is occurring (Fig. 32.2). Ultrastructural changes have been described (323). Peripheral neuropathy characterized by edema, demyelination, and axonal degeneration accompanied by marked hypertrophy and hyperplasia of neurilemmal cells may be seen with lasalocid toxicity (124).



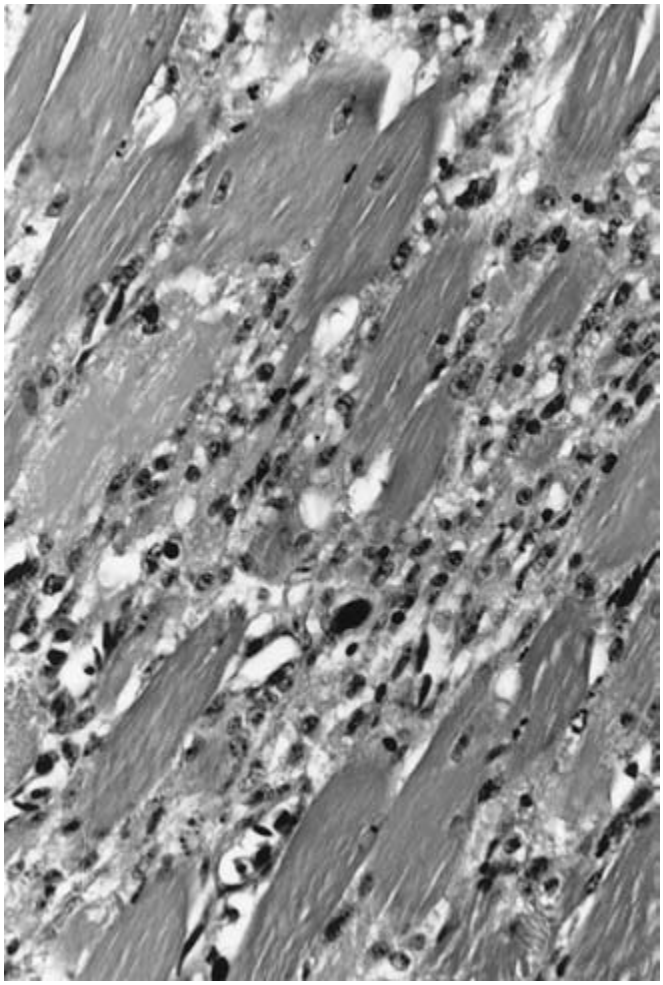
32.1. Acute ionophore toxicity. Dyspnea and drooping wings are suggestive of heat stress. (Barnes)

Differential Diagnosis

Because there is a marked individual, age, and species variation in susceptibility, and the toxic effect may be potentiated by other drugs, normal levels of ionophore should not be dismissed if clinical signs and histologic changes indicate ionophore toxicity. High serum or plasma levels of muscle enzymes may be useful in differentiating ionophore toxicity from botulism (223). Ionophore toxicity also must be distinguished from vitamin E/selenium deficiency and *Cassia (Senna)* ingestion, which may produce similar signs and lesions.

Other Anticoccidials

3,5-dinitro-*o*-toluamide (dinitrotolumide, dinitolmide, DNOT, Zoalene, Zoamix) can cause ataxia, torticollis, incoordination, and reduced growth (165, 242, 266). Nicarbazin (Nicarb) can make broiler chicks listless, dull, and ataxic; in older birds, there can be reduced egg production, shell depigmentation, yolk mottling, and reduced hatchability (18, 164, 191). Nicarbazin depresses growth rate at 150 mg/kg feed. Even when used at recommended levels, nicarbazin increases metabolic rate and heat production (20, 266, 342). This makes older broilers more susceptible to heat stress and pulmonary hypertension syndrome. Generally, there are no gross lesions, but there may be hepatic and renal epithelial degeneration (242, 267). Nitrophenide (Megasul) has caused nervous signs but with rapid recovery (242). Ducks, geese, and chukar partridges may have depressed growth and mortality from halofuginone (Stenorol) (21, 96), and reduced skin strength has been found in chickens (122, 208). Use of *t*-butylaminoethanol may result in reduced growth due to choline deficiency.



32.2. Muscle from a young turkey with knockdown. Minimal muscle necrosis and inflammation along with increased sarcolemmal or satellite cell nuclei indicate regeneration.

Antiprotozoals

Organic arsenicals and imidazoles such as dimetridazole (Nitrazol, Emtryl), used for histomoniasis, have caused growth depression, drops in egg production, nervous signs (ataxia, incoordination, tremors), convulsions, and death in geese, ducks, pigeons, and turkeys (266, 267, 270). Waterfowl may be poisoned by doses safe for other poultry. Quinacrine HCl (Atabrine), used for *Haemoproteus* infections in pigeons, was fatal at a dose of approximately 50 mg/kg.

Organic Arsenical and Imidazole Feed Additives

Phenylarsonic acids such as arsanilic acid (*p*-amino-benzene arsanilate) and sodium arsanate, roxarsone (3-nitro-4-hydroxyphenylarsonic acid), and nitarsone (4-nitrophenylarsonic acid, Histostat-50) are used to improve feed efficiency in livestock. *p*-Ureidobenzenearsonic acid (Carb-o-sep, Carbarsone) and dimetridazole (1,2-dimethyl-5-nitroimidazole, Nitrazol, Emtryl) are used for prevention and control of histomoniasis. Toxicity occurs with accidental or deliberate overdose or in dehydrated animals or birds (234). Peripheral neuropathy causing lameness in turkeys developed after they were given twice the recommended level of 3-nitro-4-hydroxyphenylarsonic acid (349). Toxicity, with liver lesions suggestive of inorganic trivalent arsenite, occurred in broilers receiving 10 times the recommended dose of this same growth promotant. Lesions may have resulted from degradation and reduction of the organic product to the trivalent state, or, more likely, from biliary excretion of inorganic arsenic present as a contaminant (287). Cysteine exacerbates toxicity, perhaps by reducing the arsenical to the more toxic trivalent state.

Signs

Ataxia and incoordination usually are seen, but stunting and depression also may be prominent signs. Lameness may be evident in turkey poults.

Pathology

Gross changes may be absent, although affected birds are usually small with an empty digestive tract. Microscopically, peripheral nerves may show loss of myelin, fragmentation of axons, and proliferation of neurilemmal cells (267, 271). Ulcerative cholecystitis occurred in turkey poults (38).

Anthelmintics

All anthelmintics are probably toxic if a sufficient overdose is given, but generally birds are more resistant than mammals to anthelmintics.

Benzimidazoles

Cambendazole, mebendazole, and fenbendazole are well tolerated by birds (273).

Imidazothiazoles

Levamisole and tetramisole are not quite as safe as benzimidazoles. The lethal dose—50% (LD_{50}) of tetramisole for chickens—is 2.75 g/kg. Geese and captive birds are more susceptible (273) with a fatal dose for captive kiwis between 25 and 43 mg/kg (116); 300 mg/kg is toxic for geese and as little as 66 mg/kg of levamisole is toxic for some wild birds. Anthelmintic activity of *dl*-tetramisole resides in the *l*-isomer (levamisole), so the effective dose of levamisole is half that of tetramisole. This doubles the safety margin. Tetramisole is no longer available in most countries. Levamisole poisoning has occurred in geese being treated for *Amidostomum* infection (355). Levamisole was toxic for ducks parenterally at 40 and 80 mg/kg (129). Microscopic lesions in kiwis killed by levamisole were similar to those of mammals consisting of pulmonary congestion, edema, and bronchopneumonia and severe periacinar cytoplasmic vacuolation of hepatocytes (116).

Organophosphates

Organophosphorus compounds have caused poisoning in birds eating treated feed intended for horses (155, 201). The resin pellet form of dichlorvos (DDVP) is toxic because it is retained in

the gizzard. Colored breeds of chickens are more susceptible than white breeds to coumaphos, and naphthaphos has a narrow safety range for chickens, with 50 mg/kg being fatal (273).

Ivermectin

Ivermectin has a wide safety margin in birds. An oral or injectable dose of 0.1 mg/kg has been suggested (273). Ivermectin is effective against a wide range of parasites. Zeman (356) tried 1.8 mg/kg for *Dermanyssus gallinae.* This dose was more effective in chickens weighing more than 450 g. The toxic dose for chickens is 5.4 mg/kg, which causes 4-hour somnolence; 16.2 mg/kg, which causes 24-hour listlessness and ataxia; 48.6 mg/kg results in death 5 hours postinjection. Canaries given 20–60 mg/bird IM showed temporary immobility.

Other Anthelmintics

Phenothiazine is relatively nontoxic for birds, and hygromycin B is safe at 8 g/900 kg feed (273).

Nutrients and Other Feed- and Water-related Toxicants

Amino Acids

Interaction among some amino acids relate to growth, but only methionine is toxic to poultry. Methionine toxicity affects chickens and quail (174, 286) and has caused depressed growth and cervical paralysis in turkey poults (128). Mortality can occur at levels of 1.8% in feed. Methionine attenuates calcium-induced kidney damage (341). Ethinone (a methionine antagonist) toxicity in chicks can be relieved by methionine.

Antinutrients

A variety of feed stuffs and potential feed stuffs are poorly digestible, contain factors that inhibit digestion (protein inhibitors), depress growth, cause pasting of feces, or increase the incidence of skeletal disorders. Antinutritional factors in some of these products (e.g., soybean and some other beans) can be destroyed by heat. The nutritional value of some feedstuffs (e.g., wheat, barley, and rye) can be improved by enzymes (34, 114, 153, 163). Antinutrients that can be found in plants include proteases, tannins, saponins, antivitamins, lectins, b-glucans, pentosans, polysaccharides, concanavalin A, hemagglutinins, vicine, convicine, alkaloids, and sinapines. Feedstuffs known to contain antinutrient factors are alfalfa (166, 322), amaranth (4), jackbeans (77, 194, 231), fababeans (233, 277), lima beans (230), narbon beans (87), soybeans (166, 196), jojoba (13), lupins (35, 248, 275), peas (36), vetch (276), barley, rye, wheat (19, 34, 209), and sorghum (316).

Protein Supplements

Fish and Meat Meals

Gizzerosine, histamine, histidine, and other biogenic amines cause digestive disturbances, stunting, and osteoporosis (152, 313). Biogenic amines result from heating or bacterial spoilage of fish and animal byproducts. Toxic products get into poultry feed through fish or meat meal. Excess acid secretion in the proventriculus is stimulated by gizzerosine, causing gizzard erosion and hemorrhage (148, 216, 273). Broiler chickens may die from hypovolemic shock. Black ingesta and blood may run from the mouth (*vomito negro*), and the contents of the digestive tract are often melanic. Other biogenic amines reduce broiler feed efficiency (177).

Minerals

For information on trace mineral deficiency and toxicity (tissue levels, signs, etc.), see references 259 and 260. Information on poultry in these references is included for the following minerals: aluminum, arsenic, cadmium, calcium, chloride, chromium, cobalt, copper, fluoride, iodine, iron, lead, magnesium, manganese, mercury, molybdenum, nickel, phosphorus, potassium, selenium, sodium, tungsten, vanadium, and zinc. More detailed information on macro and trace mineral deficiencies and excesses are reported in Chapter 29, "Nutritional Diseases."

Aluminum

Aluminum depressed growth rate in chicks, due to decreased feed intake, and decreased egg production in adults when 0.3% was added to the feed (30, 351). Aluminum also may interfere with phosphorus retention (85, 92, 157) and iron absorption resulting in anemia (131). Aluminum absorption after ingestion may be affected by the acidity of the ration (45).

Calcium

Excess absorbed calcium is excreted through kidneys; high levels cause ureter and kidney impaction, resulting in nephrosis. Very young birds are most susceptible. This condition may be produced in pullets by feed delivery mistakes where layer ration is accidentally fed to pullets. High mortality from hyperuricemia with visceral urate deposits may result from kidney damage because of high dietary calcium. Lung pathology with damage to parenchyma from calcium deposits may also occur in young chicks. It is possible that nephrosis and visceral urate deposits in young and dead-in-shell chicks may result from kidney obstruction by calcium. Excess unabsorbed calcium remaining in the intestine increases fecal water content of pullets and hens on high calcium rations. If the source of calcium is dicalcium phosphate, the alkaline solution formed in the upper digestive tract may result in epithelial necrosis (239, 242, 332), particularly if the mineral has been "top-dressed" on feed, and birds eat undiluted material.

Urolithiasis in pullet and layer flocks may be caused by high calcium and low phosphorus in pullet rations. The incidence may also be increased by infectious bronchitis virus infection (120).

Cobalt

Moderate levels (125 ppm) stimulate polycythemia and induce pulmonary hypertension. Higher levels (500 ppm) cause marked tibial dyschondroplasia, and necrosis and fibrosis in the pancreas, liver, and skeletal, smooth, and cardiac muscles. All levels reduce feed intake and growth (73).

Copper

Copper sulfate is added to water for treatment of enteritis or yeast infection or to clean algae or scum from water lines and drinkers.

Addition to feed is another method for treating enteritis and candidiasis. It also may be sprayed on litter to control *Aspergillus* or used as an antifungal preparation on wood. Birds occasionally are poisoned by eating copper sulfate crystals. Diets low in calcium may increase susceptibility to copper toxicity (189). Mortality in turkeys offered water containing copper sulfate may have resulted from dehydration caused by water refusal, rather than from copper poisoning. Toxicity signs are depression and weakness with convulsions and coma terminally (242) or anemia (140, 234, 271). Gross lesions include necrosis of proventriculus and gizzard epithelium with sloughing of koilin lining (117, 144).

Fluoride

Growth, production, and egg quality were reduced by 700 and 1000 mg sodium fluoride/kg feed (125). Leg deformity has also been described. Laying birds can tolerate ingesting 4.453 mg fluoride/day for up to 74 weeks (53).

Iodine

Reduced egg production and weight and increased embryonic mortality in the first week and at pipping occurred when 350 ppm of iodine was added to the ration of turkey breeder hens (51). With experimentally induced iodine toxicity in chickens, researchers found that clinical signs were poor growth and a bizarre syndrome of chicks falling over, lying motionless, getting up, and then repeating falling over (16).

Magnesium

Excess magnesium causes bone abnormalities by replacing calcium and affecting phosphorus utilization (190).

Phosphorus

Excess phosphorus affects growth plate development of bones and increases tibial dyschondroplasia and leg deformities. Phosphate may also be caustic to moist oral and epithelial surfaces. White phosphorus induces mortality and hematologic abnormalities after oral ingestion (306, 307).

Potassium

Potassium in the form of fertilizer or potassium permanganate is toxic. The latter causes epithelial necrosis of the digestive tract (242).

Sodium (Sodium Chloride, Sodium Bicarbonate)

Excess ionic sodium, usually from sodium chloride in feed or water, causes significant economic losses in poultry in many countries. Most toxicity results from consuming saline water; not water deprivation. Sodium in feed can be toxic for young chicks and poults with or without water deprivation. In some cases of toxicity at apparently low salt levels, analysis may have been for chloride, with salt level calculated from chloride level. When Na^1 toxicity is suspected, both feed and water should be analyzed for Na^1; not estimated from chloride content. There may be sources of Na^1 in feed or water other than sodium chloride. Levels of Na^1 in feed and water are additive. Sources of sodium may also be naturally occurring in soil or water (210).

Young birds are much more sensitive to Na^1 toxicity than are adults, probably because their kidneys are not yet fully developed (211). Water with Na^1 greater than 0.4% (4000 ppm) is quite toxic and will cause high mortality within a few days. Lower levels may be toxic as well, depending on the amount of Na^1 in feed. Levels of Na^1 greater than 0.12% (1200 ppm) are toxic for some chicks and poults and produce heart failure with edema and ascites. Feed with Na^1 greater than 0.85% is toxic for some chicks and poults. Much lower levels will cause heart failure and ascites even when water is available freely. Because steroids increase Na^1 and water retention (285), resulting in hypervolemia, hypertension, right ventricular failure, and ascites, stress may also contribute to Na^1 susceptibility. Birds have poor renal concentrating ability and difficulty reducing plasma osmolality by excretion of salt in excess of water. Some waterfowl have nasal salt glands, which allow them to excrete Na^1 if an excess is ingested.

Three forms of disease result from Na^1 toxicity in young birds. At high levels, birds develop acute, severe diarrhea and dehydration, lose weight, and die. There is often acute kidney damage, particularly with sodium bicarbonate (212), which may be ischemic because of increased red blood cell rigidity. Potassium may have a protective effect (301). At lower levels, loose droppings also occur, but birds gain weight, at least for 1–2 days, because of associated water retention. Depending on the Na^1 level, they may subsequently eat less and grow poorly, or continue to eat and grow well. Water retention, with hypervolemia and reduced red blood cell deformability (214), can lead to functional cardiac overload, causing marked right ventricular hypertrophy and dilation, valvular insufficiency, edema, and ascites in chicks (168, 169, 215). At intermediate levels of excess sodium, a variety of clinical signs and pathologic changes are seen, depending partly on how long birds survive with hypertension before heart failure occurs and how long they survive afterward. Many lesions described for Na^1 can be attributed to heart failure. The severity of ascites may be affected by other dietary, environmental, and water constituents (279, 297).

Signs. At low levels of excess Na^1, only watery droppings are seen until ascites occurs. At this stage, chicks and poults are dyspneic, depressed, and have a swollen abdomen. At high Na^1 levels, birds are obviously sick and depressed within a few hours, with thirst and diarrhea. They may have rough, dirty, wet feathers or down. Nervous signs may be present, and some birds may be prostrate. At intermediate levels, stunting of some birds may be prominent. Excess Na^1 may cause reduced egg production and increased mortality in adults (65).

Pathology. Chicks with ascites and edema frequently have excess fluid in lungs and hydropericardium. Young males may have cystic dilation of seminiferous tubules (271). There is cardiac hypertrophy, which in chickens is mainly right-sided. Poults have biventricular hypertrophy with dilatory cardiomyopathy. At levels of Na^1 causing dehydration, the following also may be seen: cyanosis, myocardial hemorrhage, nephrosis, and enteritis.

Microscopic lesions are frequently secondary to heart failure or dehydration. For a detailed description of histologic lesions,

see reference 217. Glomerulosclerosis (285, 301) may be ischemic in origin. Ultrastructural changes in heart muscle (232) include glycogen accumulation, myofibrillar disarray, Z-band streaming, and disruption of intercalated discs.

Sulfate

The toxic concentration of sulfate is affected by age of the birds, source (water or feed), other salts, etc., and is not clearly defined. Magnesium sulfate may be more toxic than sodium sulfate (325). Diarrhea, reduced growth, and depressed egg production can occur.

Selenium

Some plants accumulate selenium (347), and the addition of acceptable treatment levels of selenium to rations already containing high normal selenium levels may produce toxicosis. This often manifests itself as embryo deformities of the eye, head, or beak in the progeny from such breeder flocks (228). Decreased growth and feed intake resulted when there was 4–8 ppm selenium in drinking water (44), but the toxicity of selenium varies with the form present (149). Selenium can accumulate in the food chain of aquatic birds causing emaciation, hepatitis, and ascites (123).

Zinc

Toxic levels of zinc (>500 ppm) cause anorexia, depressed growth, reduced egg production, gizzard and pancreatic lesions, and hematologic abnormalities (66, 70, 175, 198, 202, 300, 343). Individual birds may be poisoned by ingesting metallic zinc, such as coins or other objects, or galvanized wire from caging in the case of pet birds (268) and in wild waterfowl via contaminated mining areas (300).

Metals and Metalloids

Arsenic

Inorganic, aliphatic, and trivalent organic arsenicals are used as pesticides, weed and brush killers, and defoliants. Toxic effects include diarrhea, nervous signs, and cyanosis. There is inflammation of the digestive tract including crop, proventriculus, and gizzard, hepatosis, and nephrosis (234, 287). Most reports of arsenic toxicity in birds are experimental, except those associated with grasshopper bait (242). For information on organic arsenicals, see "Antimicrobials, Anticoccidials, and Growth Promotants."

Cadmium

Toxic levels of cadmium found in industrial waste and sewage sludge cause decreased feed intake, decreased growth, induced kidney lesions, and reduced gonadal mass and function (154, 257, 259, 260, 340). Experimental cadmium toxicity in chicks, poults, and ducklings and free radical-induced lesions by cadmium, silver, and other minerals have been reported (25, 60, 324).

Chromium and Potassium Dichromate

Chromium from industrial waste or coated metal objects may cause depression, anorexia, and paralysis (154, 259, 260).

Lead

All species of birds are susceptible to lead poisoning. Lead is the only metallic poison causing significant disease in birds, and most toxicity occurs in wild species, especially waterfowl. Chickens are more resistant than waterfowl (259, 260). Birds as a group are at risk from metallic lead because the material is retained in the gizzard, ground down, and absorbed slowly. Experimental poisoning trials with chickens show an interaction with some nutrients (82, 167), production of immunotoxic effects with differential gender sensitivity (40), inhibition of avian bone healing (177), and immunosuppression (354).

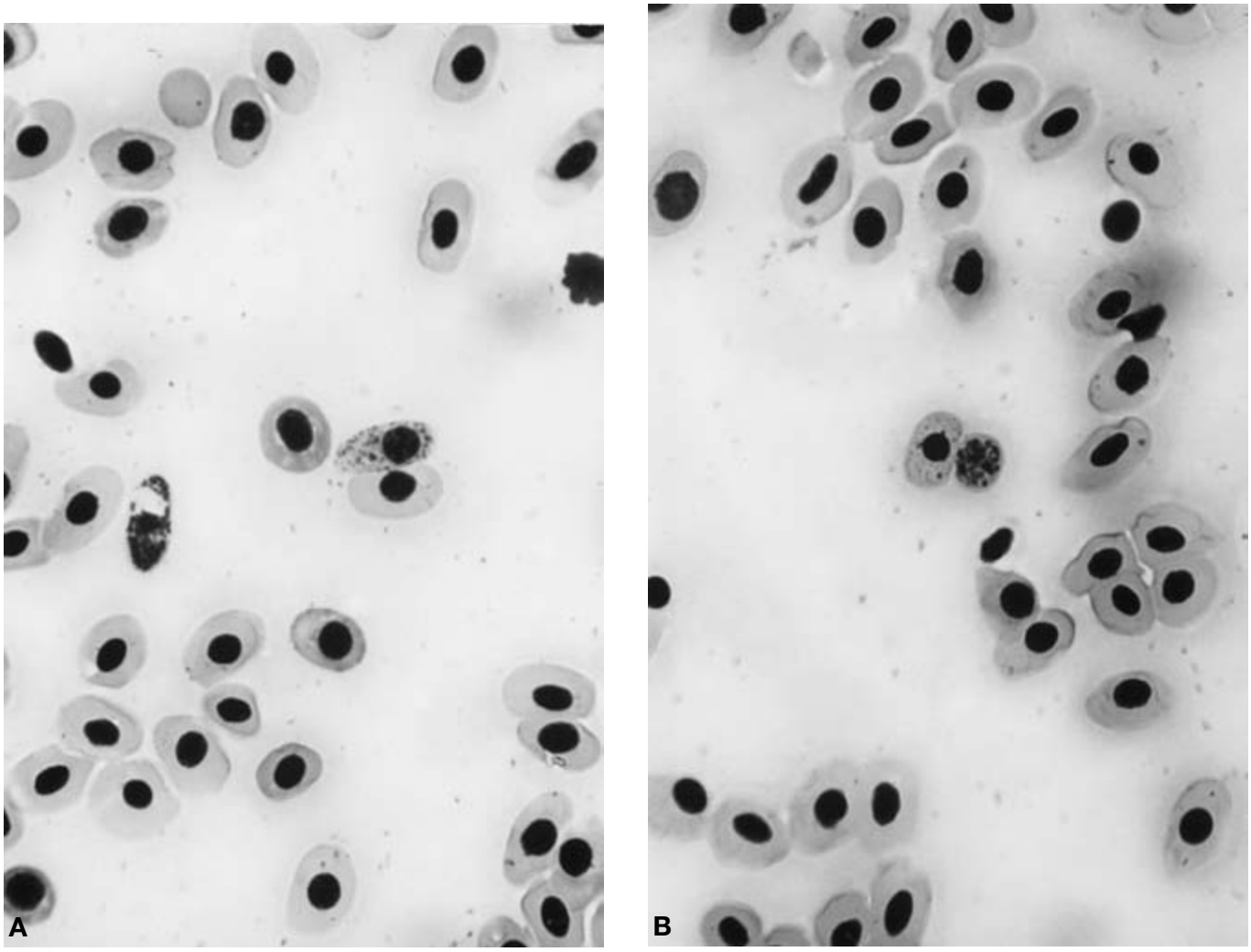
Lead is widespread in the environment, and there are many possible sources for ingested lead when toxicity occurs. Wild water birds are at greatest risk from ingesting lead shot or contaminated sediments (150), which is the main hazard in North America (282) and elsewhere (139). Lead weights from fishing lines are the most important source in England. Pigeons may also ingest lead shot (69). Birds that eat carrion may be poisoned by lead shot ingested with tissues. Backyard and free-range poultry may pick up lead from paint chips, lead batteries, or other lead objects. Chicks have been poisoned by eating contaminated grit (242). Cage birds may be poisoned from the same environmental sources as children and dogs—mainly paint chips, leaded windows, toys, and lead objects (353).

Signs. Most lead poisoning in birds is chronic. Clinical disease usually is seen as wasting, ataxia, lameness or paralysis, and anemia. In acute cases, anorexia, weakness, prostration, and anemia may be prominent. Green diarrhea may result from anorexia, or it may be a direct effect of lead on digestive and nervous systems.

Hematology. Basophilic stippling and abnormal erythrocytes may occur in lead-poisoned birds but are not present in all affected birds (Fig. 32.3) (242). Finding anemia with mitosis of erythrocytes and large numbers of immature cells may be more significant.

Pathology. Most lesions probably result from anorexia and debility. Emaciation may be prominent, but many ducks and geese that die from lead poisoning are in good body condition. The carcass may be pale with watery blood. Erosion and ulceration of the gizzard lining can be extensive (Fig. 32.4). Impaction of proventriculus frequently is seen and is likely secondary to vagus nerve damage (Fig. 32.5).

Microscopically, the most diagnostic lesions are demyelination of peripheral nerves and focal areas of vascular damage in the cerebellum (156), and acid-fast, intranuclear inclusion bodies in the kidney (Fig. 32.6), liver, and spleen (199, 242, 271). Inclusions are composed of protein-bound lead and can be demonstrated by special staining or electron microscopy (Fig. 32.7) (227). Nephrosis with degeneration and necrosis of tubular epithelial cells containing brown pigment have been described. Hemosiderosis is prominent in the spleen and other organs. Scattered myocardial necrosis associated with hyaline or fibrinoid necrosis of blood vessels (172), arrested mitotic activity in proventricular epithelial cells, and degenerative changes in testes also may be found (206).



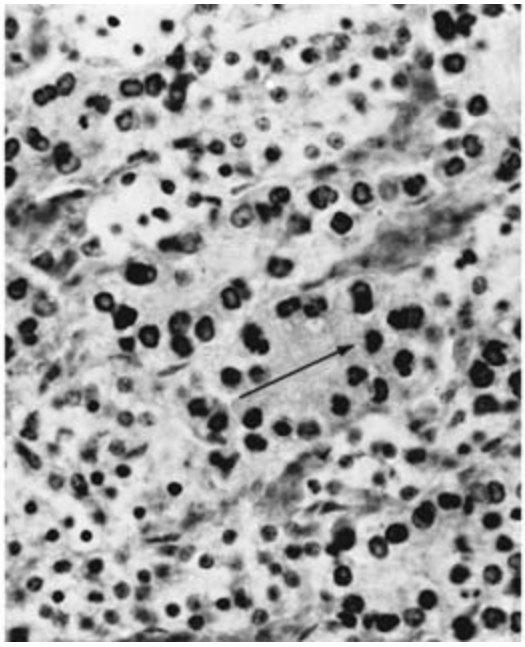
32.3. Duck with lead poisoning. A. Immature erythrocytes and two cells showing basophilic stippling. (Barnes) B. Basophilic stippling in an erythrocyte adjacent to an immature erythrocyte undergoing mitosis. (Barnes)



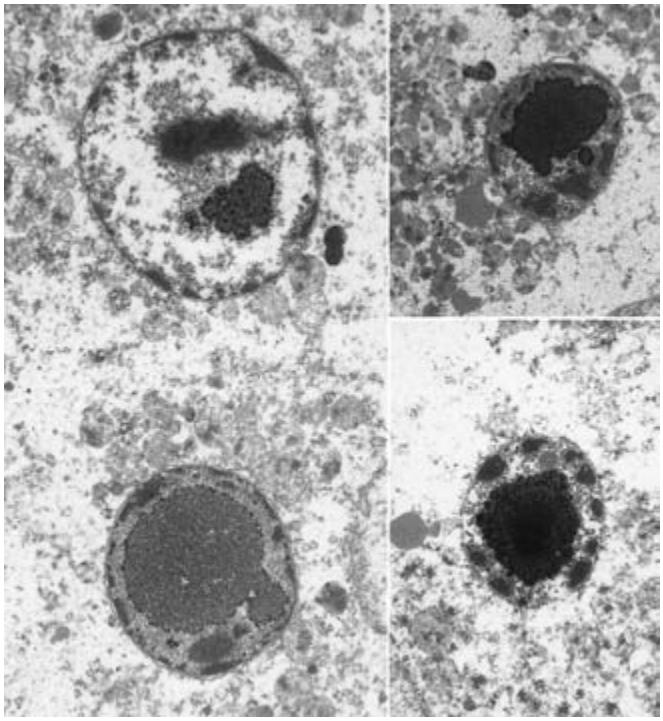
32.4. Gizzard from duck with lead poisoning. Severe erosion, ulceration, and bile staining of koilin lining. Note 2 lead pellets retrieved from the gizzard. (Barnes)



32.5. Lead poisoning, showing distended proventriculus (arrow); there were 15 leads shot in the gizzard.



32.6. Lead poisoning. Acid-fast intranuclear inclusion bodies (arrow) in kidney of mallard duck. ×480 (Locke)



32.7. Proximal renal epithelium from a bird with lead poisoning. Nuclei contain irregular, variable electron-dense inclusion bodies typical of lead accumulation in kidney. Similar inclusions may be present in the liver. (Shivaprasad)

Diagnosis. The final diagnosis of lead poisoning is based on blood and tissue levels. In chickens, a blood lead level greater than 4 ppm, a liver lead level greater than 18 ppm wet weight, or a 20 ppm wet weight in kidney would be considered diagnostic (259, 260). Lead levels in bone can also be determined. Acid-fast inclusions in kidney epithelial cells suggest lead poisoning but may be found in birds that could have ingested lead but died from some other cause. Peripheral nerve lesions, in conjunction with fibrinoid necrosis of blood vessels, which may be found throughout the body not just in brain and heart, are useful in diagnosis, but similar changes are seen in methylmercury poisoning (271). In lead poisoning, however, lesions in the central nervous system are related only to vascular damage.

Mercury

Organic mercury, used previously as a seed protectant, is discussed later in this chapter along with fungicides. Most organic mercury in the environment today results from methylation by aquatic organisms and action of methogenic bacterial enzymes on elemental mercury from nature (decaying trees) or industry. Tons of mercury as bivalent inorganic mercury, elemental mercury, and phenyl mercury have been discharged into waterways around the world.

Methylmercury, a direct product of biotransformation, gets into small water organisms and enters the food chain when fish eat contaminated plants, insects, or animals (bioconcentration). Fish-eating birds, particularly ducks, may become poisoned from mercury in the food they eat (234). Mercury contamination of pheasants also has occurred. Experimental feeding of low levels of methylmercury resulted in decreased egg production, increased shell-less eggs, and reduced hatchability (234).

Residues in chickens given subclinical amounts of methylmercury were highest in liver, least in muscle, and intermediate in kidney. Eggs had 4 times as much mercury in albumin compared with yolk (234).

Inorganic mercury of medicinal or industrial waste origin may induce anorexia, enteritis, and nephrosis (234, 259, 260).

Tin

Tin from medicinal sources can cause depression, hunching up, and yellow diarrhea (295).

Uranium (Uranyl Nitrate)

Industrial uranium causes depression, anorexia, and nephrosis with severe lesions in collecting tubules, followed by hyperuricemia and visceral urate deposits in birds that survive (184).

Vanadium

Vanadium can contaminate phosphorus sources and cause reduced egg quality, growth, and hatchability (183, 259, 260). Also, there are many reports in the literature of experimental vanadium toxicity.

Vitamins

Vitamin A

Excess vitamin A reduces egg production (180) and growth rate and causes osteodystrophy and osteoporosis (315, 326).

Vitamin D_3 (Cholecalciferol)
Four percent mortality due to kidney failure occurred in chicks when feed was top-dressed with vitamin D_3 powder. Nephrosis with focal mineralization was present throughout the kidneys. Mineralization was also present in the walls of arteries, particularly arteries in the proventriculus. Excess vitamin D_3 has resulted in increased incidence of leg abnormalities in broilers (57). Experimentally induced toxicity indicated that 25-hydroxycholecalciferol was 100 times as toxic as cholecalciferol (265). A variety of lesions were seen, but renal damage was most significant (219). Poultry, pigeons, and wild birds may also be poisoned by rodenticides in which the toxic agent is 25-hydroxycholecalciferol.

Vitamin B_6 (Pyridoxine)
Pyridoxine is toxic for pigeons at levels safe for poultry (90–100 mg/bird, i.e., approximately 200 mg/kg body weight given by injection) (244).

Other

Ethoxyquin
Ethoxyquin (1,2-dihydro-6-ethoxy-2,2,4-trimethylquinolone) is a commonly used antioxidant that may be toxic at high levels (6500–12,500 mg/kg feed). Mortality is increased; affected birds have pale, swollen kidneys; dark-brown enlarged livers; and urates in joints. Proximal tubular necrosis in the kidney and accumulations of dark-brown pigment, interpreted to be ethoxyquin, in hepatocytes, bile ducts, and pulmonary blood capillaries are seen microscopically (195).

Lignosol
Calcium lignosulfonate, a pellet binder, may produce black, sticky cecal contents that adhere to the skin of processed broilers, causing increased condemnation from contamination. It has no effect on body weight or feed conversion (258).

Nitrate and Nitrite
Nitrate is converted to nitrite by bacteria in the digestive tract and is much less toxic than nitrite. High levels of nitrate cause diarrhea, dyspnea, and death. Lower levels affect growth and egg production. Blood hemoglobin is changed to methemoglobin. The effect is greater in young birds, as it is in young mammals, with fetal hemoglobin (74). Most reports of toxicity are experimental, although there are some reports of toxic nitrate levels in leaves and stems of plants (345).

Pen- and Litter-related Toxicants

Pen and litter-related toxicants include products accidentally or intentionally incorporated into litter or applied to the pen, that result in illness. Some are disinfectants and fumigants discussed later in this chapter. Except for boric acid, insecticides mixed into the litter (e.g., fire ant control products) or applied to walls, floor, or ceiling are covered later in this chapter. Copper sulfate, often used as a fungicide in litter, has been discussed with feed- and water-related toxicants above. Toxic mixtures, such as copper-chrome-arsenic formulations, are used as preservatives in the timber industry (225). Occasionally, part of the building structure is toxic; geese have been poisoned from eating urea-formaldehyde foam insulation picked from the wall.

Boric and Orthoboric Acid

Boric acid is used in litter to control darkling beetles and may be consumed by broilers, which results in reduced growth and abnormal feathering (82, 274).

Iron

Ferrous sulfate heptahydrate added to litter to reduce ammonia formation was toxic to broilers (331). Affected chicks were depressed and lethargic. Those that died had severe gizzard ulceration and liver degeneration. The LD_{50} of ferrous sulfate is 7010 mg/kg body weight for a single dose. When added to the diet, 3% caused reduced growth and feed intake, and 1.5% had no effect (249).

Pentachlorophenol

Pentachlorophenol has been used as a pesticide in industry and agriculture, but its primary use is as a wood preservative. Logs may be treated before they leave the forest, or wood may be treated after cutting. Sawdust and shavings from treated wood frequently have been used as poultry litter; chickens can become contaminated from contact with these shavings. Because the product is used for many other purposes, pentachlorophenol may also contaminate broilers or table eggs in other ways.

Illness associated with pentachlorophenol has been caused by toxic impurities such as dioxins (see later discussion). Pure pentachlorophenol can reduce growth, cause kidney hypertrophy, and decrease humoral immune responses (256, 309). It has also been associated with musty taste in eggs and broiler meat. Chlorophenols in litter are metabolized by bacteria and fungi to chloroanisoles. Anisoles have a musty or earthy odor even at very low concentrations, and they are responsible for the taste in eggs and meat from chickens in contact with contaminated litter (112). Reduced hatchability has also been associated with pentachlorophenol contamination (115).

Sulfur

Elemental sulfur may be sprinkled on dirt floors and vaporized by adding water and heating the building before litter is put down. High mortality, ulcerative dermatitis mainly affecting moist areas of the body, irritation of respiratory mucous membranes, and conjunctivitis occurred in chicks placed in treated buildings (251). Lesions probably resulted from sulfur dioxide (from residual sulfur that had not vaporized) dissolving in moisture on the chick's body to form sulfurous acid (H_2SO_3).

Disinfectants and Fumigants

Fumigants are products producing toxic gases used to control rodents, insects, fungi, and bacteria. They can cause toxicity when

inhaled or ingested. Phenolic disinfectants can be toxic when inhaled or absorbed through skin.

Phenolic Compounds and Coal-Tar Derivatives

Phenol, cresol, creolin, carbolineum, and creosote products cause damage to vascular endothelium, epithelia of respiratory and digestive tracts, and parenchymal organs, such as liver and kidney (242). Thymus and bursa of Fabricius are small, but this may result from stunting rather than being a direct effect on the immune system. Hydropericardium is prominent, but ascites and subcutaneous edema are also frequently present if contact is severe. Mortality may be high. Diagnosis is based on a history of contact and by elimination of other causes of ascites and edema. Odor may also provide a useful clue. Cases of creolin toxicity still appear in the literature (193). Coal tar poisoning has been induced in ducks by feeding from clay pigeons (48).

Quaternary Ammonium (Cationic Detergents)

Use of sanitizers to clean poultry drinkers or treat water has resulted in reduced growth or production and, occasionally, severe lesions and death in young chicks (158). High levels of quaternary ammonia cause epithelial irritation of the mouth, pharynx, and upper respiratory tract, resulting in oral, ocular, and nasal discharges. Necrosis of epithelium leads to pseudomembranes in the mouth and epithelial thickening in the esophagus, crop, and proventriculus, with ulcers at the gizzard-proventricular junction (72, 242). Similar lesions have been reported in poults (205).

Chlorine

Low levels (37.5–150 mg/kg) may have a beneficial effect, but high levels (300–1200 mg/kg) result in reduced growth and increased mortality (63).

Formaldehyde

Formaldehyde gas and formalin (a 37% solution of the gas in water, which is then 100% formalin) have been widely used for many years as antibacterial and antiviral agents in the poultry industry. Photophobia and respiratory signs from contact with high levels of formaldehyde are seen occasionally in newly hatched or recently delivered baby chicks and poults. Prolonged exposure to high levels of formaldehyde, which dissolves in liquids on mucous membranes to produce formalin, in the hatcher impairs ciliial function and causes tracheal epithelial degeneration and sloughing (281). Air quality during subsequent growout, however, has a greater effect on productivity than does early formaldehyde exposure (280). Epithelial necrosis of eyes, mouth, and trachea with pseudomembranous plaques in the mouth and trachea also may be found. Edematous swellings under the lower beak (118), subcutaneous edema (27) during the acute phase, and ascites or edema occurred later in exposed poults.

Other Fumigants

It must be assumed that most or all chemicals used as fumigants are toxic to poultry (234). A few reports of deliberate or accidental poisoning of poultry by other fumigants appear in the literature (294, 339).

Fungicides

Fungicides are used as seed dressings (protectants), wood preservatives, in paint and plastic, and on cereal crops, fruits, vegetables, and flowers. Previously, poisoning in poultry usually has resulted from the incorporation of treated seed into poultry feed.

Organic Mercurials

Mercurial fungicides, frequently ethyl or methyl mercuric chloride, that cause poisoning with central and peripheral nervous lesions in poultry, wild birds, animals, and humans consuming treated seed are no longer in use (142, 143, 271, 305). Signs of organic mercury poisoning may be nonspecific, or affected birds may show progressive paralysis or other neurologic signs. Specific gross lesions may be lacking, but microscopically, Wallerian degeneration of peripheral nerves and spinal cord, and neuronal damage in the brain may be present. Vasculitis may also be obvious in some vessels, particularly in the brain.

Thiram

Arasan (active ingredient thiram, a dithiocarbamate) has caused poisoning in poultry, producing lameness and leg deformity in chicks and poults and soft-shelled eggs in layers (126, 242). It is also teratogenic (236). Thiram increases the incidence and severity of tibial dyschondroplasia (89). The LD_{50} is 485–932 mg/kg body weight in pheasants and 2800 mg/kg body weight in mallard ducks (62).

Captan

Captan is an organic seed protectant. It is less toxic than Arasan. It depresses feed consumption, slows growth, and reduces egg production (242).

For descriptions of other organic synthetic fungicides, see reference 234; pentachlorophenol, a widely used wood preservative, and copper sulfate, a litter treatment, have been covered previously in this chapter.

Herbicides

Chlorates

Sodium and potassium chlorates used as herbicides and defoliants are moderately toxic for poultry. They act by converting hemoglobin to methemoglobin. The lethal dose for chickens is 5 g/kg (234).

Organic Synthetic Herbicides

Amitrate (3-amino 1,2,4-triazole) causes hypothyroidism and reduces weight gain in chickens (350). Phenoxyherbicides, such as 2,4-D, cause kidney enlargement. Some herbicides are toxic for embryos (84). See reference 234 for additional information.

Dipyridyl Herbicides (Diquat and Paraquat)

Paraquat toxicity results from free radical induced membrane damage caused by the inhibition of the glutathione peroxidase system. Selenium is protective. Experimental oral paraquat poisoning in turkeys produced diarrhea, listlessness, and anorexia

with terminal convulsions. Gastroenteritis was present at necropsy (274). Turkeys are more resistant than are mammals (140, 234), but exposure of mallard duck eggs produces cranial and pelvic deformities at hatch (278).

Insecticides

Insecticides may be referred to by either their common or registered name. The common name is not capitalized (e.g., carbaryl), but the trade name is (e.g., Sevin) (234). Organic insecticides (organophosphates, organochlorides, and carbamates) have been widely used, some on animals and birds as systemic larvicides and anthelmintics, as well as on buildings and pens. Wild birds have been poisoned by feeding on treated animals (32, 134). Many insecticides are quite toxic for animals as well as insects, arthropods, and helminths. Some more toxic products are used on crops, wood, trees, as soil insecticides, and as seed dressings. Extensive tables presenting general information on insecticides can be found in (234).

Organochloride Insecticides

The mode of action of organochloride (chlorinated hydrocarbon) insecticide toxicity is unknown. Generally, they act to diffusely stimulate or depress the nervous system. Organochloride insecticides often remain longer in the environment than other insecticides. Some more persistent ones have been taken off the market or have restricted use. Because they are fat soluble, organochlorides tend to build up in the food chain and be present in yolks of eggs. There is considerable literature available on insecticide toxicity in wild birds, with emphasis on organochlorides (218).

Signs. Nervous signs varying from excitement with vocalization to tremors, ataxia, and convulsions are prominent. Prostration and death may occur without other signs. Other signs include salivation, vomiting, diarrhea, and depression. Lameness and leg deformity may also occur. There may be decreased egg production, a drop in hatchability, embryo mortality, loss of pigment on pigmented eggs, a change in shell texture (chalky), and eggshell thinning. Administration of atropine to acutely affected birds will not alleviate or modify signs of toxicity.

Pathology. Specific lesions do not occur in organochloride toxicity. Nonspecific changes such as congestion and hemorrhage may be present. Excess cerebrospinal fluid may be noted when the brains of affected birds are examined.

Chlordane
Chicks develop ataxia and hyperexcitability; hens have reduced body weight, decreased egg production, atrophy and cyanosis of the comb and wattles, and hydropericardium (242).

DDT and DDE
Hens develop tremors, production drop, and weight loss, and there is eggshell thinning (242).

Dieldrin
Pigeons, gulls, and other birds may show nervous signs (9, 242).

Heptachlor
This may cause ataxia, salivation, prostration, and death (241, 330).

Lindane
Diarrhea, vomiting, anorexia, depression, convulsions, and sudden death have been associated with lindane poisoning (28, 242).

Mirex
This has caused embryo mortality (3).

Toxaphene
Lameness, thin shells, and osteomalacia may occur (238, 242).

Organophosphorus and Carbamate Insecticides

These products inhibit acetylcholinesterase, causing acetylcholine to accumulate, which results in the overstimulation of parasympathetic nerves and muscles (98). Atropine given to effect will often dramatically reverse clinical signs, but efficacy depends on the type of organophosphorus compound and the duration of the intoxication and amount of time lapsed between exposure and treatment. Repeated treatments may be necessary. Some organophosphates and carbamates have delayed neurotoxic effects (see the following discussion). Chickens and other birds are more susceptible than mammals to this type of toxicity.

Signs. Chicks and poults may die quickly, showing few signs except dyspnea and paralysis, or they may exhibit lacrimation, salivation, diarrhea, tremors, depression, dullness, lethargy, cyanosis, ataxia, incoordination, and convulsions prior to death. Because of respiratory signs and salivation in early stages, respiratory infection may be suspected initially.

Pathology. Few gross lesions occur. There may be congestion with dark blood, and hemorrhages may be present in heart muscle, on serosal surfaces, and on mucosa of intestines. No specific microscopic changes have been identified.

Diazinon
Diazinon is used to control fire ants and darkling beetles, but in birds, diazinon can cause incoordination, paralysis, respiratory signs, and death (147). It is also used to control pests in soil and grass, causing death in Canada geese (113, 308).

Dichlorvos (DDVP)
Dichlorvos induces staggering, frothing from the mouth, paralysis, and convulsions (91).

Dimethoate
Toxic effects include reduced growth and egg production (290, 291).

Famphur
Mortality in raptors has resulted from famphur toxicity (145).

Fenthion
Laying hens exposed experimentally had neurologic deficits followed by decreased egg production and reduced body weight (321).

Malathion
Malathion causes dullness, salivation, loose droppings, cyanosis, paralysis, and death; lesions include injected subcutaneous vessels and dark congested heart (37, 242). In geese, there may be flaccid paralysis.

Monocrotophos
Monocrotophos toxicity has been associated with salivation, mortality in quail, and weight loss and embryo abnormalities in chickens (296).

Parathion
Parathion induces lacrimation, salivation, dyspnea, tremors, and convulsions (242).

Carbamates
Various carbamates such as carbaryl, carbofuran, and others are toxic for pheasants, pigeons, turkey poults, chickens, and ducks (14, 91, 263). Signs include reduced growth, lameness, weakness, ataxia, and death. There may be tibial dyschondroplasia, retarded testicular development because of degeneration of seminiferous epithelium, nerve fiber degeneration, and congestion of organs and tissues.

Delayed Organophosphorus Neurotoxicity

Delayed neurotoxicity occurs several days to weeks after exposure, causing progressive degeneration of the peripheral nerves and spinal cord, which leads to weakness and paralysis. Acetylcholinesterase is not affected. Delayed neurotoxicity may result from ingestion or absorption of a variety of triaryl phosphates; chemicals found in phenylphosphorothioate insecticides such as leptophos, cyanofenphos, and their analogues; as well as a variety of industrial chemicals, including fire retardants and lubricants. Malathion and dimethoate may also cause delayed neurotoxicity. Mature chickens, pheasants, and mallard ducklings are highly susceptible (234). Chicks hatched after *in ovo* exposure also show clinical signs (99). There are many reports of delayed neurotoxicity in chickens from these products. Most describe experimentally induced lesions (1, 2, 86, 186, 200, 335). A previous author of this chapter had seen turkeys in Ontario, Canada, with typical clinical signs of ataxia and paralysis and histologic lesions of delayed organophosphorus neurotoxicity in spinal cord and peripheral nerves. Clinical cases occurred in Europe after chickens ate scraps of synthetic leather containing tri-ortho-cresyl-phosphate (TOCP) (242).

Signs. Ataxia, falling sideways, inability to rise, lack of leg reflexes, and prostration may be evident. Birds appear bright and eat and drink if given access to food and water for several days.

Pathology. There are no gross lesions. Degeneration of axons and myelin in peripheral nerves and long tracts of the spinal cord are diagnostic. Axons may be swollen, and spheroids may be present in axon spaces. Digestion chambers containing macrophages and debris may be present in subacute cases.

Other Insecticides

Pyrethrum and Synthetic Pyrethroids
These products are not very toxic to animals or birds, and there are no reports of illness (52).

Rotenone
Rotenone (derris powder) is prepared from roots of *Derris* spp. Mature chickens are relatively resistant (lethal dose 1000–3000 mg/kg); young birds are more susceptible (234). Fish are very susceptible to rotenone.

Nicotine
Nicotine sulfate (Black Leaf 40) has been used to paint chicken roosts to control insects and arthropods, particularly northern fowl mite. It has also been used for internal parasites. In low doses, nicotine stimulates the nervous system through an acetylcholine-like activity. At toxic levels, neural transmission is blocked causing death from respiratory paralysis (234).

Signs. Sudden death, occasionally preceded by depression and coma, is seen in affected birds.

Pathology. Because death is from respiratory failure, cyanosis and congestion may be marked. Hemorrhages may be present on the heart and in other tissues.

Rodenticides, Avicides, and Molluscacides

Rodenticides

Information on cholecalciferol and arsenic is presented in "Nutrients and Other Feed- and Water-related Toxicants."

Alpha-naphthyl Thiourea (ANTU)
ANTU causes depression, anorexia, weakness, prostration, and death. Lesions include pulmonary edema, hydropericardium, fatty change in liver, and myocardial degeneration.

Sodium Monofluoroacetate (Compound 1080)
Signs are reluctance to move, edema of wattles, dyspnea, cyanosis, and nervous signs. Lesions include dark, unclotted blood, pulmonary hemorrhage and edema, clotted blood in the trachea and air sacs, petechiation, enteritis, and hydropericardium (140, 234).

Strychnine
Toxic effects are tonic spasms, respiratory failure, reproductive failure, and increased mortality of progeny from exposed hens (234, 242, 243, 352).

Warfarin, Brodifacoum, and Diphacinone
These anticoagulant rodenticides are sold under a variety of trade names and may be combined with sulfaquinoxaline to interfere with vitamin K synthesis. They inhibit epoxide reductase, which converts vitamin K to its active form. Toxicity causes anemia with fluttering, gasping, and hemorrhages in eyes, mouth, and

other tissues (15, 140, 234, 292). Onset may be rapid, and death occurs within 72 hours of ingestion (221). With the long-acting anticoagulants, there is a cumulative effect, and if a small amount of anticoagulant is consumed repeatedly, there may be little or no product found in the digestive tract.

Phosphorus
Elemental yellow, red, and white phosphorus can induce depression, anorexia, diarrhea, ataxia, gastroenteritis, and death (234, 242, 306, 307).

Zinc Phosphide
Weakness, diarrhea, opisthotonos, and convulsions occur. There is enteritis, ascites, and hydropericardium (135, 292). In an accidental poisoning of broiler breeder chickens, birds were found dead following ingestion of zinc phosphide treated oats used as a rodent bait. No clinical signs were observed. Gross lesions consisted of hydropericardium and ascites. A petroleum-like odor, common feature with this poison, of crop contents was detected. Histologic lesions were those of congestion of many internal organs, along with severe pulmonary edema and congestion. (320) Wild birds may also succumb to this poison if they are allowed access to zinc phosphide treated grain (254).

For birds, the toxic doses of several rodenticides (a-chloralase, crimidine, pyriminil, phosphorus, a-chlorohydrim) are given in *Clinical and Diagnostic Veterinary Toxicology* (234).

Avicides

Avitrol (4-aminopyridine or 4-AP)
Avitrol causes disorientation and vocalization (distress calls). Affected pigeons may be molested by normal pigeons. Generalized congestion is present at necropsy, and the characteristic small pellets usually can be found in the ventriculus (111, 222).

2-chloro-4-acetotoluidine (CAT) and 3-chloro-P-toluidine (CPT)
No clinical signs have been described, but kidney necrosis (from CAT) and liver and kidney necrosis (from CPT) occur (119).

Molluscacides

Metaldehyde
Nervous signs were prominent in ducklings following ingestion of metaldehyde (11).

Toxic Gases

Ammonia

Ammonia levels should be less than 25 ppm, but in poorly ventilated litter-type houses, ammonia may exceed 100 ppm (171). High levels of ammonia (50–75 ppm) reduce food consumption and growth rate (67). Egg production is also reduced. Ammonia dissolves in the liquid on mucous membranes and eyes to produce ammonium hydroxide, an irritating alkali causing keratoconjunctivitis. If levels greater than 100 ppm persist, corneal ulceration and blindness can occur. The condition is painful, and photophobia and stunting are marked. At levels of 75–100 ppm, changes in respiratory epithelium include loss of cilia (235) and increased numbers of mucus-secreting cells (8). Heart rate and breathing may be affected, and there may be hemorrhages in trachea and bronchi. For a review, see reference 47.

Carbon Monoxide

Carbon monoxide (CO) poisoning may occur in buildings where defective or unventilated gas-catalytic or open-flame brooders or furnaces are in use, or where poultry are exposed to internal combustion-engine exhaust fumes. Affected chicks or poults show drowsiness, labored breathing, and incoordination. Spasms and convulsions may occur prior to death. At postmortem, blood is bright red. Sublethal levels cause stunting (242, 310). In suspected cases, CO should be measured at several locations in the pen with the ventilation system shut off. Carboxyhemoglobin can be measured in the blood of affected birds. A previous author of this chapter found levels of 70 ppm CO in pens where a repeated high incidence of ascites due to pulmonary hypertension and right ventricular failure occurred. Toxic levels of CO for chickens are as follows: 600 ppm for 30 minutes causes distress; 2000–3600 ppm is lethal in 1.5–2 hours (234).

Aerial Endotoxin

Breakdown of bacteria in the litter and environment results in endotoxin in the air in broiler pens. Endotoxin from inhaled air can be found in the blood of people who work in broiler pens (79). The effect of inhaled endotoxin on poultry is not known.

Polytetrafluoroethylene

Fluorinated gases are released when this material, used as a nonstick coating (Teflon) on light bulbs, cookware, and ovens, is overheated. Pet birds that inhale the fumes die from lung edema and hemorrhage. Histologic examination reveals epithelial necrosis in tertiary (para)bronchi and vascular damage in blood capillaries (311, 337).

Other Toxic Gases

Levels of methane, carbon dioxide, hydrogen sulfide, methyl mercaptan, dimethyl sulfide, and dimethyl disulfide were found to be low in poultry and other livestock buildings in Finland (171), but toxic fumes from liquid manure pits in pig barns have killed humans and pigs in North America, and nitrogen dioxide formed in freshly filled silos has also killed humans and animals in Canada and the United States. Toxic gases associated with livestock production, including poultry, have been reviewed (234). The effect of sulfur dioxide in chickens has been described (100).

Household and Commercial Products

Alcohol

Ethyl alcohol may be used to dissolve experimental chemicals or drugs given to poultry in feed or water. Clinical signs of intoxication include ataxia and reduced feed consumption. Fatty change in the liver and heart lesions may occur (7, 59). Wild birds frequently are intoxicated from ingesting fermented fruit

(103). Pet birds may obtain alcohol inadvertently, or it may be given by the owner.

Antifreeze (Ethylene Glycol)

Ethylene glycol is toxic when ingested because it breaks down to oxalic acid, which combines with calcium to form calcium oxalate. Calcium oxalate crystals block renal tubules and cause tubular epithelial necrosis leading to hyperuricemia and urate nephrosis with visceral urate deposits. Diagnosis usually is based on finding typical crystals and tubular changes on microscopic examination of kidney (261, 271, 272, 312). Liver necrosis is found in pigeons. Other forms of toxicity may occur in other species (234). Coccidia oocysts treated with ethylene oxide were toxic to chicks and caused kidney lesions similar to ethylene glycol (338).

Carbon Tetrachloride

Carbon tetrachloride has been used previously as a household solvent and cleaner. It has also been used to treat tapeworms in chickens (273). It is toxic to animals and birds, interfering with fat metabolism and causing liver and kidney damage. Chickens are more resistant than rats, but low levels cause decreased growth.

Fertilizer

Lawn, garden, and farm fertilizers contain nitrogen, phosphorus, and potassium. The contents in that order usually are given by numbers representing each element as a percentage of the total. These elements have been discussed previously. Fertilizer may be attractive to birds because it is frequently in the form of small hard pellets. Some phosphate fertilizers contain very low levels of radioactive material.

Naphthalene

Mothballs frequently are recommended to keep pets and other animals away from gardens or out of attics. They have also been used in chicken nests for ectoparasite control. Mothballs are toxic and can cause poisoning in poultry and pet birds (181).

Urea

Urea is relatively nontoxic for birds. Because it is used in feed preparations for ruminants, the pellets occasionally are found in poultry feed.

Industry-Related Toxicants

Toxic Fat Syndrome, Chick Edema Disease, and Dioxin Toxicity

More than 30 years ago, the most toxic dioxin, 2,3,7,8-tetrachlorodibenzodioxin (TCDD), and other dioxins in the same polychlorinated dibenzodioxin group were found as contaminants in industrial fat (tallow from cattle hides) added to poultry feed. This material, which could be distilled from fat, was called "chick edema factor" until it was identified. It caused widespread disease in the broiler industry and in other poultry for several years. Occasional cases of dioxin toxicity (chick edema disease) occurred until about 1970. More recently, TCDD toxicity followed environmental contamination in Italy where adult fowl died with lesions of chick edema disease (253). Dioxin was probably the material causing lesions in toxicosis caused by paint containing chlorinated hydrocarbon (207).

Chickens are more susceptible than some mammals to the toxic effects of TCDD (234). In chickens, dioxin damages vascular endothelium causing vascular leakage and extensive movement of fluid into body cavities and subcutaneous tissue. The epithelium of some parenchymal organs is damaged, and there is degeneration of heart and skeletal muscle.

Because right ventricular hypertrophy and dilation have been described (6), and many lesions in dioxin toxicity are similar to right-sided heart failure from other causes, the possibility of dioxin contributing to right ventricular failure should be considered.

Depending on the level of TCDD in feed, variable numbers of broilers in a flock will show severe signs of stunting, respiratory distress, weakness, ataxia, and edema. Mortality occasionally can be very high. For a description and review of the syndrome, see reference 242.

TCDD may be present as a contaminant in herbicides. It and other dioxins are produced by incineration (234) and by industry (93). Contaminated poultry carcasses potentially could serve as a route for human exposure, because dioxins are retained in body fat after exposure.

Polybrominated Biphenyl (PBB) and Polychlorinated Biphenyl (PCB)

PBBs or PCBs accidentally may be added to feed, get into feed as contaminants (as in oil or grease from equipment), or be present in the environment from industrial contamination and deliberate dumping. Both PBBs and PCBs are toxic to birds. At low levels, they reduce production, reproduction, hatchability, offspring viability, and thyroid hormone levels (109). Hepatocyte damage and bursal depletion also occur with low-level PBB toxicity (65). Exposure of young embryos magnifies these effects (107). Residues may be found in eggs and meat from birds without clinical signs. PBBs are concentrated in eggs at 1.5 times the dietary level (93). At high levels, lesions of PCB toxicity are similar to dioxin toxicity. It is likely that in some cases, PCBs were contaminated with TCDD.

Crude Petroleum and Oils

Most information on toxicity of oil to birds deals with environmental contamination and the effect of oil spills on waterfowl. Ingested oil causes anorexia, weight loss, incoordination, tremors, and Heinz body anemia. Lesions include lipid pneumonia, enteritis, hepatosis with fatty infiltration, and nephrosis and degeneration of pancreas, spleen, and bursa (226). Immune responses are impaired. In herring gulls and puffins, lesions suggested a primary toxic hemolytic disease (191) with lymphoid depletion being secondary and stress related. Oil applied to chicken eggs caused embryo mortality and lesions in organs (55). Oils and oil products on the feathers and skin can be removed with detergents.

Biotoxins

Biotoxins are poisonous substances produced by living organisms including bacterial toxins such as botulinum toxin, which in birds is frequently associated with toxin-contaminated maggots, bacterial food poisoning, diseases such as necrotic and ulcerative enteritis, gangrenous dermatitis, and mycotoxins. Perhaps, even methylmercury produced by bacteria should be classed as a biotoxin. Insect and snake venoms (188) are also biotoxins. Most of these conditions are of little importance or are discussed in other parts of the text. Botulism is being seen with increasing frequency in housed turkeys and broilers. Birds are very susceptible to botulinum toxin and may show clinical signs following ingestion of very small amounts. The toxin apparently develops in dead birds left in the litter. Birds may pick up toxin from eating decaying tissue or contaminated fly larvae, darkling beetles or litter. An enzyme-linked immunosorbent assay (ELISA), which may be as sensitive as the mouse-inoculation test, has been developed to identify botulinum toxin (see also Chapter 22). Only algae poisoning and rose chafer toxicity are mentioned here.

Algae

Several species of blue-green algae produce a toxin that, when concentrated by rapid algal growth (bloom) in warm bodies of fresh water and a constant light wind blowing the toxic material to the side of the lake, may poison animals and birds consuming it. Toxicity varies directly with concentration (159). Affected chickens may show nervous signs and paralysis before death. Ducks and turkeys have also been poisoned (154). Cyanosis, congestion, and a dilated, distended heart may be seen at necropsy (242). The liver is swollen with necrosis of hepatocytes. Diagnosis is based on identifying toxin in the water (234).

Rose Chafers

Rose chafers (*Macrodactylus subspinosus*) are insects appearing in spring and early summer in eastern and central North America. Young chicks may be poisoned by 15–30 insects (242). Clinical signs include drowsiness, weakness, prostration, and convulsions (242).

Phytotoxins

All or parts of some plants are toxic, or if fed at low levels may only reduce growth rate. Antinutrients are discussed in the section "Nutrients and Other Feed- and Water-Related Toxicants." For additional information on plants that are toxic to poultry and pet birds, see references 12, 76, 78, 83, 108, 176, 298, 347.

Avocado (Persea americana)

Part of Plant
Fruit.

Signs and Lesions
Muscle degeneration, hydropericardium, and subcutaneous edema (41, 136).

Black Locust (Robinia pseudoacacia)

Part of Plant
Leaf.

Signs and Lesions
Depression and paralysis; hemorrhagic enteritis.

Bladder Pod (Sesbania [Glottidium] vesicaria)

Part of Plant
Seed.

Signs and Lesions
Diarrhea, cyanosis, prostration, necrotic enteritis, and gizzard ulceration (95).

Cacao (Theobroma cacao, Theobromine Toxicity)

Part of Plant
Bean waste.

Signs and Lesions
Acute cases, nervous signs followed by convulsions and death; cyanosis, cloacal prolapse, and mottled kidneys. Chronic cases, anorexia and diarrhea (242).

Cassava (Manihot spp. Cyanide, Polyphenols)

Part of Plant
Root (tuber).

Signs and Lesions
Sudden death. Depressed growth (94, 240).

Carolina Jessamine (Gelsemium sempervirens)

Part of Plant
All.

Signs and Lesions
Progressive muscle weakness, seizure activity and death. No lesions. (319)

Castor Bean (Ricinus communis)

Part of Plant
Bean.

Signs and Lesions
Progressive paralysis with prostration (like botulism); diarrhea, emaciation, swollen pale mottled liver, hemorrhagic catarrhal enteritis, petechiae, degeneration of lymphoid tissue and parenchymal cells of liver and kidney, bile duct proliferation (162, 229, 242).

Coffee Senna, Sickle Pod (Cassia occidentalis, C. obtusifolia, Senna occidentalis)

Part of Plant
Seed.

Signs and Lesions
Weakness, ataxia, paralysis, decreased egg production, diarrhea; toxic myopathy; pectoralis and semitendinous muscles pale; and edematous, muscle degeneration, and necrosis (105, 237, 242, 302, 327). There is also primary axonal damage producing a neuropathy that adds to the paresis (42, 137). Lymphoid populations in the spleen and bursa of Fabricius may also be decreased (303).

Corn Cockle (Agrostemma githago, Githagenin Toxicity)
Part of Plant
Seed.

Signs and Lesions
Depression, rough feathers, decreased respiratory and heart rate, diarrhea, depressed growth; hydropericardium; caseous necrosis of crop, pharyngeal mucosa, and mouth (146, 242).

Cotton Seed Meal (Gossypol Toxicity)
Signs and Lesions
Cyanosis, inappetence, emaciation, reduced egg production and quality; enteritis, degeneration of liver and kidney, and reduced growth (242).

Coyotillo (Karwinskia humboldtiana)
Part of Plant
Fruit and seed.

Signs and Lesions
Depressed growth, cyanosis, and paralysis.

Crotalaria spp. (Pyrrolizidine Alkaloids, Monocrotaline Toxicity)
Part of Plant
Seed, leaf, and stem.

Signs and Lesions
Dull, inactive, reduced feed consumption, stunting, bright yellow-green urates; subcutaneous edema, ascites, hydropericardium, lung edema, hepatitis, bile duct hyperplasia (5, 49, 75, 242, 346).

Daubentonia (Daubentonia longifolia, Sesbania drummondii, S. macrocarpa)
Part of Plant
Seed.

Signs and Lesions
Weakness, depression, stunting, diarrhea, emaciation; proventriculitis with ulceration and enteritis; liver and kidney degeneration (104, 106, 203, 289).

Death Camas (Zygadenus spp.)
Part of Plant
Leaf, stem, and root.

Signs and Lesions
Weakness, salivation, diarrhea, and prostration (202).

Eucalyptus cladocalyx (Cyanide or Prussic Acid)
Part of Plant
Leaf.

Signs and Lesions
Acute death without premonitory signs.

Hemlock (Conium maculatum, Conine Toxicity)
Part of Plant
Seed.

Signs and Lesions
Salivation, weakness, nervous signs, paralysis, diarrhea, reduced growth; hepatic congestion, enteritis (110).

Jimsonweed (Datura stramonium, D. ferox, Scopolamine, Hyoscyamine)
Part of Plant
Seed.

Signs and Lesions
Reduced growth (182).

Leucaena leucocephala (Mimosine Toxicity)
Part of Plant
Leaf containing mimosine.

Signs and Lesions
Depressed growth, osteopathy (141, 170).

Lily of the Valley (Convallaria majalis)

Milkweed (Asclepias spp. Asclepidin Toxicity)
Signs and Lesions
Weakness and incoordination, convulsions, prostration, leading to death or recovery (242).

Nightshade (Solanum nigrum, Belladonna Toxicity)
Part of Plant
Immature fruit.

Signs and Lesions
Dilated pupils, incoordination, prostration (133).

Nitrate (Numerous Plant Species)
See previous discussion on nitrates and nitrites.

Oak (Quercus spp., Tannin Toxicity)
Part of Plant
Leaf.

Signs and Lesions
There is severe diarrhea, anorexia, and increased water consumption; enteritis, swollen kidneys, and visceral gout; diffuse necrosis of proximal renal tubules (179).

Oleander (Nerium oleander, Glycosides)

Part of Plant
All parts.

Signs and Lesions
Depression, weakness, diarrhea; gastroenteritis, liver degeneration, death (242, 314).

Onions, green (Allium ascalonicum)

Part of Plant
All parts.

Signs and Lesions
Sudden mortality. Epicardial hemorrhage and palor, hydropericardium, and hepatosplenomegaly. Microscopic lesions of hemosiderin in heptocytes, Kupffer cells and renal tubules. Onions are known to produce a Heinz body anemia in animals but was not found in geese. (56)

Oxalate (Numerous Plant Species, Oxalic Acid)

Part of Plant
Leaf and stem.

Signs and Lesions
Oxalate nephrosis (345). See also the previous discussion on ethylene glycol.

Parsley, Ammi majus, Others (Photosensitization)

Part of Plant
All parts.

Signs and Lesions
Dermatitis (unfeathered areas); hepatitis (245, 293).

Pokeberry (Phytolacca americana)

Part of Plant
Fruit.

Signs and Lesions
Ataxia, leg deformity, ascites (17).

Potato (Solanum tuberosum, Solanine Toxicity)

Part of Plant
Green or spoiled tubers, peelings, and sprouts.

Signs and Lesions
Incoordination, prostration (teratogenic) (133, 317).

Ragwort (Senecio jacobea Pyrrolizidine Alkaloid)

Part of Plant
All parts.

Signs and Lesions
Focal hepatic necrosis and portal fibrosis (49).

Rapeseed Meal (Erucic Acid/Glucosinolate Toxicity; Antinutrients Sinapine, Tannin, Phytic Acid); Canola at Low Glucosinolate Levels

Part of Plant
Seed.

Signs and Lesions
Abnormal odor and taste in eggs, hypothyroidism, depressed growth, anemia, sudden death, ruptured liver, hepatitis, ascites, hydropericardium; periacinar hepatic necrosis, fatty change in skeletal and heart muscle (22, 26, 43, 54, 101, 121, 173, 264, 344).

Sweet Pea (Lathyrus spp., Lathyrism)

Part of Plant
Seed (pea).

Signs and Lesions
Skeletal deformity, osteolathyrism (*L. odoratus*); or neurologic disease, neurolathyrism (*L. sativus*) (50, 220, 262).

Tannins (Numerous Plant Species)

Tannins are antinutrients that occur in a variety of plants. It may be important to determine tannin levels in some feedstuffs such as sorghum (161, 316, 333).

Tobacco (Nicotiana tabacum, Nicotine Sulfate Toxicity)

Part of Plant
Leaf and stem.

Signs and Lesions
Stunting, reduced production (teratogenic) (242).

Velvetweed (Malvaceae Family, Cyclopenoid Fatty Acids)

Part of Plant
Seed.

Signs and Lesions
Pasty, rubbery yolk in eggs (178).

Vetch (Vicia spp., Cyanogenic Glycoside)

Part of Plant
Seed (pea).

Signs and Lesions
Excitability, respiratory distress, convulsions (138, 269).

Yellow Jessamine (Gelsemium sempervirens)

Part of Plant
Whole plant.

Signs and Lesions
Depressed growth (242, 348).

Yew (Taxus spp., Taxine Toxicity)

Part of Plant
All parts.

Signs and Lesions
Labored breathing, incoordination, and collapse; cyanosis.

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VI Other Diseases

33 Emerging Diseases and Diseases of Complex or Unknown Etiology

Chapter 33

Emerging Diseases and Diseases of Complex or Unknown Etiology

Introduction

Y. M. Saif

The emergence of new diseases and the re-emergence of recognized diseases are familiar events in the annals of poultry medicine. Some of these emerging diseases could have been present earlier but were not recognized because of low prevalence, mild signs and lesions, lack of diagnostic techniques, or misdiagnosis. In other situations, genetic changes in the microorganisms could have rendered them more virulent or pathogenic. Similarly, genetic changes in the bird could have altered its susceptibility and resistance to disease. In addition, changes in environmental conditions or management could result in conditions that are favorable for a microbe to express pathogenic properties. Because of the global activities of the poultry industry, resulting in the continual movement of live birds, eggs, and poultry products across political borders, it is difficult to contain an emerging or re-emerging disease to a country or a continent. Hence, it is necessary to maintain a vigilant attitude toward poultry health and to sustain capable diagnostic facilities.

There are disease conditions that have multifactorial etiologies including combinations of microbes and, at times, microbes plus nutritional or environmental factors. Examples abound of seemingly harmless microbes that do not cause disease in healthy individuals but can become pathogenic following an insult, although it might be mild, to the host. *Escherichia coli* is a prime example of such a microbe, earning it a designation as a universal secondary infection in poultry. In the commercial poultry environment, viruses and bacteria, including some that have the potential of causing disease, are common. Live vaccine viruses, some of which are very mild pathogens, may also be present.

In addition, flocks that are immunocompromised because of infectious or noninfectious agents could present unusual disease syndromes, increased susceptibility to disease, or lack of responsiveness to vaccination. The combinations of etiologies of disease could result in additive or synergistic effects. The pathogenesis of the multiple etiologies is not completely understood, but some mechanisms have been suggested or shown to occur.

The upper respiratory tract and the gastrointestinal tract are bombarded continually by a variety of microbes; yet, disease is not necessarily a common event. Natural and acquired defense mechanisms function efficiently to eliminate infections, inhibit replication, or prevent colonization of tissues by microbes. The mucociliary apparatus of the respiratory tract is a highly efficient system for the elimination of microbes and particulate matter. Some viral infections result in the deciliation of parts of the respiratory tract and lysis of infected cells, resulting in accumulation of cellular products and debris, creating an environment favorable for bacterial multiplication and attachment to cells, which are important events in the pathogenesis of bacterial infections. In the gastrointestinal tract, similar events initiated by viruses have been described, including villous atrophy and consequent increased bacterial replication and adherence to cells. It is a common finding in respiratory and enteric diseases of poultry to encounter a variety of infectious agents. Because of the possible complex etiology of respiratory and enteric disease, it is important to understand the role of the different agents in the disease process. Such understanding should be helpful in designing logical control or prevention strategies.

This chapter was first introduced in the 10th edition with the realization that there will be continuous change in its content. Some of the diseases recognized as emerging at a point of time become either established entities or fail to have a sustained impact. In either case, the outcome will be the removal of these diseases from this chapter.

In this chapter, information is presented on the complexity of respiratory and enteric diseases. In addition, the newly recognized diseases are described.

Two subchapters, Big Liver and Spleen Disease and Hepatitis Splenomegaly Syndrome that were included in this chapter in the 11th edition were moved to Chapter 14 of this edition. This follows the recognition that both designations refer to one clinical entity. In addition, the work of Meng *et al.* elucidated the etiology of the condition being hepatitis E virus.

Poult enteritis–mortality syndrome is included in the subchapter on Multicausal Enteric Diseases in recognition of the etiology of the condition being multifactorial and to avoid confusion in nomenclature.

Multicausal Respiratory Diseases

Stanley H. Kleven

Although much is known about the individual agents responsible for respiratory diseases in poultry, uncomplicated infections with single agents are the exception. Under commercial conditions, complicated infections involving multiple etiologies with viruses, mycoplasmas and other bacteria, immunosuppressive agents, and unfavorable environmental conditions are more commonly observed than simple infections. In addition, respiratory reactions induced by routine vaccination programs may themselves play a major role in the development of respiratory disease.

Interactions among Respiratory Pathogens

Perhaps the best understood examples of multiple respiratory infections are those involving mycoplasmas; this subject has been reviewed previously (10, 32). Although uncomplicated *Mycoplasma gallisepticum* infections in turkeys ordinarily result in respiratory signs, sinusitis, and airsacculitis, simple infection with *M. gallisepticum* or *M. synoviae* in chickens often results in mild or even subclinical disease. Interactions with Newcastle disease (ND) virus or infectious bronchitis virus (IBV) are known to increase the severity of *M. gallisepticum* infection (3, 13, 16, 51, 60, 67, 74). Similar interactions also occur with *M. synoviae* (28, 29, 34, 37, 65, 71).

The virulence of respiratory viruses may influence the severity of mycoplasma infections. With *M. synoviae,* concurrent challenge with high-passage field and vaccine strains of IBV resulted in milder respiratory disease than did exposure to field strains (28), and chicken-passaged vaccine virus resulted in more severe airsacculitis than did the original vaccine when birds were concurrently challenged with *M. synoviae* (29).

The timing of exposure to infectious agents is important in the pathogenesis of complicated infections. Generally, respiratory virus and mycoplasma infection must occur concurrently or within a short period of time for synergism to occur (29, 37), but mycoplasma-free chickens had a milder clinical response to IBV challenge than did chickens that were chronically infected with *M. gallisepticum* (67).

Other infectious agents are also known to interact with *M. gallisepticum.* Synergistic effects between *Avibacterium paragallinarum* and *M. gallisepticum* are well known (4, 33, 39, 48); control of *M. gallisepticum* results in milder respiratory disease due to infectious coryza. Interactions are also known between *M. gallisepticum* and adenovirus (5), reovirus (31), and laryngotracheitis (9).

Three-way interactions between vaccine virus (Newcastle disease virus [NDV] and/or IBV), mycoplasma (*M. gallisepticum* or *M. synoviae*), and *Escherichia coli* resulted in more severe respiratory disease than any two alone. Combinations of any two of the agents resulted in milder disease than the three-way combination, and challenge with only one of the individual agents resulted in very mild or no disease (46, 65). Chickens exposed to IBV and *M. gallisepticum* did not become susceptible to *E. coli* until 8 days postchallenge (24).

Interactions with various agents have also been described for *M. meleagridis* in turkeys. Enhanced airsacculitis was observed when germ-free poults were challenged with *M. meleagridis* and *E. coli* (61). An interaction between *M. meleagridis* and *M. synoviae* has been noted for turkey sinusitis (56) but not for airsacculitis (57). Combination infections with *M. meleagridis* and *M. iowae* caused more severe airsacculitis than either agent alone (57). *M. gallinarum,* ordinarily considered to be nonpathogenic, induced airsacculitis in broilers when given in combination with ND/infectious bronchitis vaccine virus (36).

Pathogenicity is increased when infectious bronchitis is combined with *M. imitans* (20); *M. imitans* also shows a similar effect when combined with rhinotracheitis virus in turkeys (21).

Interactions between *E. coli* and other respiratory agents often occur in the absence of mycoplasma infection. Exposure to *E. coli* or IBV alone resulted in little or no clinical signs or mortality, but challenge with various strains of IBV along with *E. coli* resulted in significantly increased clinical signs and mortality (52, 63, 77). Such a combination challenge with *E. coli* provided a means of evaluating the protection induced by infectious bronchitis vaccine strains against various challenge strains (12, 52). However, Newcastle disease vaccination nonspecifically stimulated resistance against *E. coli* at 2–8 days postvaccination, as measured by the number of *E. coli* recovered from the spleen (30). Turkeys exposed to the LaSota strain of NDV had a decreased tracheal mucus transport rate and reduced tracheal clearance of *E. coli* (19). A highly significant positive correlation between the number of infectious agents encountered during primary exposure and the incidence of colibacillosis after *E. coli* challenge was demonstrated (54). Studies on interactions between *E. coli* and *Bordetella avium* in turkeys have shown that *B. avium*-infected turkeys had higher numbers of *E. coli* in their tracheas and less ability to clear *E. coli* from the tracheas and lungs than did birds free of *B. avium* (69, 70). *B. avium* also adversely affected vaccinal immunity of turkeys to *Pasteurella multocida* (59).

Other respiratory agents may also interact; for example, the pathogenicity of Newcastle disease was enhanced when occurring in combination with *Ornithobacterium rhinotracheale* (68).

Effects of Immunosuppressive Agents

Immunosuppressive agents, especially infectious bursal disease in chickens and hemorrhagic enteritis virus in turkeys, are well known to affect adversely susceptibility to respiratory infections. Challenge of chickens with infectious bursal disease virus has been shown to affect adversely antibody response and resistance to ND (18, 22, 23, 27), infectious bronchitis (22, 53, 75), *M. synoviae,* (22), and *Aspergillus flavus* (50). “Intermediate” vaccine

strains of infectious bursal disease virus were highly variable in their interference with development of ND antibodies following ND vaccination (42).

Specific-pathogen-free chickens infected with infectious bursal disease virus and *E. coli* and then challenged with various adenovirus strains developed respiratory signs and lesions, and those infected with infectious bursal disease virus and *E. coli* without adenovirus did not (14). Infectious bronchitis virus exacerbated the infectious bursal disease virus-induced suppression of opsonization by *E. coli* antibody in chickens (47). Control of infectious bursal disease in the field is an essential factor in controlling respiratory disease in broilers.

The association between signs and mortality of colibacillosis in turkeys and the presence of hemorrhagic enteritis lesions and viruses in the spleen led to the hypothesis that hemorrhagic enteritis infection often exacerbates colibacillosis (64). This association was subsequently proved in laboratory challenge studies (40). Inclusion of hemorrhagic enteritis virus along with *E. coli* challenge has been useful for the induction of colibacillosis in laboratory studies (49). Control of hemorrhagic enteritis is considered to be an essential factor in controlling colibacillosis in turkeys.

Although Marek's disease herpesvirus is well known as an immunosuppressive agent, its role in respiratory disease has not been extensively studied. Chickens infected with Marek's disease virus, however, did not respond as well serologically to *M. synoviae* as chickens that were not challenged (35). Marek's disease virus infection may also enhance the virulence of *Cryptosporidium baileyi* (1, 2).

Chicken infectious anemia virus is also recognized as an immunosuppressive agent that may be a risk factor in respiratory and other diseases (26) and may be involved with interfering with vaccinal Newcastle disease antibody production in the field (55).

Role of Environmental Factors

It is evident that environmental factors play a significant role in interacting with infectious agents in the production of respiratory disease in poultry, but there are relatively few published studies of such interactions. Environmental factors that have been studied include atmospheric ammonia, dust, and temperature. Chickens and turkeys continuously exposed to 20 ppm of ammonia showed gross or histologic signs of damage after 6 weeks of exposure, and exposed chickens were more sensitive to infection with NDV (7). Turkeys exposed to 10 or 40 ppm of ammonia had deterioration of their normal mucociliary apparatus, excessive mucus production, matted cilia, and deciliation in tracheal tissue (44) and exhibited impaired clearance of *E. coli* from air sac, liver, and lung (45). Chickens exposed to 70 or 100 ppm of ammonia for 4 days exhibited increased thickness of atrial walls and shrinking air capillaries in their lungs (6). Ammonia levels of 25 or 50 ppm resulted in reduced body weights, feed efficiency, larger lung size, and increased airsacculitis in chickens challenged with IBV (38).

Atmospheric dust has also been shown to have a detrimental effect on the response to respiratory infections. Atmospheric dust significantly increased the severity and incidence of air sac lesions in turkeys with high or low rates of infection with *M. meleagridis* (8). In a broiler house study, losses from colisepticemia generally peaked 1 week after coliform numbers peaked in dust samplings (11). More microscopic lesions in the bronchi were observed when chickens were kept at 27°C than at 16°C. It was postulated that this may have been due to mouth breathing rather than the higher dust levels in the air of the warmer rooms (58).

Hatchery fumigation of day-old chicks may damage the tracheal epithelium (15, 62), possibly increasing susceptibility to early respiratory disease.

Respiratory disease and airsacculitis condemnations are well known to increase during the winter months, but there have been few studies on the effects of temperature on susceptibility to respiratory disease. Chickens challenged with *M. synoviae* and IBV had more extensive air sac lesions when housed at temperatures of 7–10°C than when housed at 29–24°C or 31–32°C (76).

A common observation is that increasing "down time" between broiler flocks has a beneficial effect on reducing respiratory and other diseases. In a recent field study in Delmarva, larger flock sizes were associated with increased early respiratory disease, and increased down time decreased early respiratory disease (66).

Vaccination Reactions

Protection of chickens and turkeys against viral respiratory diseases is dependent upon the widespread use of live respiratory virus vaccines. The live respiratory virus vaccines that have been most widely used are ND vaccines and infectious bronchitis vaccines. Among the live ND vaccines, there are naturally occurring apathogenic strains such as VG/GA, Ulster, and QV4, naturally occurring mild respirotropic lentogenic strains such as B_1 and LaSota, and very limited use of mesogenic strains. Live infectious bronchitis vaccines are typically chicken embryo-passaged field IBV isolates. Chicken-embryo passage is used to attenuate the virulence of virulent field IBV isolates. A single IBV isolate, for example Massachusetts 41, may be available as a live vaccine at several different embryo passage levels (virulence attenuation levels).

All of the live respiratory viral vaccines replicate in the bird and cause some degree of cell damage. The clinical manifestation of this viral replication, and its resultant pathology, is called the "vaccination reaction." The live respiratory virus vaccines are intended to induce an immune response while inducing only minimal pathology or a minimal vaccination reaction in a healthy bird in a good environment. A normal vaccination reaction for IBV or NDV should become clinically apparent 3–5 days after vaccination and should persist for an additional 3–5 days. If the vaccination reaction appears clinically to be unusually severe or prolonged, it is often referred to as a "rolling" vaccination reaction or, more simply, as a severe vaccination reaction.

Severe or prolonged vaccination reactions following the use of live ND or infectious bronchitis vaccines are a very common occurrence in the commercial poultry industry. Most typically, flocks that undergo a severe vaccination reaction develop respiratory col-

ibacillosis. The pathogenesis of this complex disease interaction follows the same pattern described for the interaction of virulent wild respiratory viruses with *E. coli* (63, 77). Most poultry health specialists agree that respiratory disease that results from the interaction of viral respiratory vaccine viruses with *E. coli* is the most common respiratory disease of commercial poultry.

Several different sets of circumstances can culminate in a severe respiratory vaccination reaction, although regardless of the inciting factors, severe respiratory reactions typically culminate in the development of respiratory colibacillosis. Immunosuppression has been demonstrated to enhance the ability of pathogens to induce disease (18, 22). Likewise, immunosuppression can impede a bird's ability to limit replication of a respiratory virus vaccine, allowing a severe vaccination reaction. Several modified live virus vaccines containing infectious bronchitis virus interfere with the ability of the gland of Harder and head associated lymphoid tissues to respond to antigenic stimulation (43). Short-term nonspecific immunity against *E. coli* induced by intravenous inoculation of inactivated bacteria or silver nitrate is suppressed by cold stress or by treating with corticosterone (41).

Vaccinating birds with respiratory virus vaccines whose respiratory tracts are contaminated with other pathogens can produce a severe vaccination reaction. The most noted examples are *M. gallisepticum* and *M. synoviae* (10). Birds contaminated with *B. avium* or *E. coli*, however, fit a similar pattern. Newly hatched chicks that have hatched in an environment heavily contaminated with *E. coli* may develop severe respiratory reactions when vaccinated at a young age with live ND or infectious bronchitis vaccines.

Some live ND, infectious bronchitis, and infectious laryngotracheitis vaccines may become more virulent if allowed to spread from bird to bird (17, 25, 29). This vaccine "backpassage" can occur in commercial poultry houses if only a portion of the birds are provided with an immunizing dose of the vaccine and the remaining birds in the house become infected by spread of vaccine virus. This type of vaccine reaction appears to be both prolonged in duration and of increased intensity.

Environmental factors can influence the intensity of a vaccination reaction. As discussed previously, ammonia and dust can interact with respiratory pathogens to enhance the severity of disease (38). This interaction is similar with respiratory vaccine viruses. Improper application of viral respiratory vaccines can enhance the severity of vaccination reactions. Spray application with a very fine spray can allow access of vaccine virus to the deep respiratory tissues and result in excessive viral replication in lungs and air sacs in addition to eliciting a stronger immune response (72). Aerosol vaccination also resulted in more severe airsacculitis after challenge with *M. synoviae* (73). Improper application of vaccines in the drinking water can prevent all birds in a house from receiving an immunizing dose of vaccine, thus providing an opportunity for spread of vaccine virus with a concomitant increase in virulence of the virus.

Because respiratory vaccine viruses of various virulence levels are available, it is important to use the proper vaccine for specific conditions. Typically, very young birds are vaccinated with highly attenuated vaccine viruses, and less attenuated vaccine viruses are used in older birds and in birds that have been previously immunized. Severe vaccination reactions can occur, for example, if chicks are vaccinated at the hatchery with a live ND or infectious bronchitis vaccine intended for use in older birds.

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Multicausal Enteric Diseases

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Introduction

The intestinal tract is a highly complex system that has numerous functions and integrates with other body systems and their functions. The intestinal tract provides the mechanism(s) by which the body can derive nutrition from its environment while simultaneously safeguarding the host. The enteric tract itself serves as an environment for other living organisms. The pathogenesis of an enteric disease is a complex entity. Likewise, the etiology of an enteric disease may be as equally complex. Combinations of, and interactions between, different viruses, bacteria and other infectious and noninfectious agents may be necessary to elicit an enteric disease or increase / decrease its severity. Similarly, combinations of, and interactions between, the intestinal tract and other body systems may not only increase / decrease the severity of enteric disease but may play key roles in the treatment, alleviation or modulation of the disease process.

Etiologies

Rarely, is a single agent the sole contributing factor of an enteric disease. Although an agent may be identified / diagnosed as the primary agent or cause of an enteric disease, other agents and factors usually contribute to the pathogenic process of the diseased intestine. Similarly, when an agent is isolated and introduced into a susceptible host under experimental conditions, the resulting disease may not be representative of the same infection caused under field conditions. Many viral agents have been reported to infect poultry, and many have been associated with enteric disease conditions. The reader is referred to Chapter 12 for a review of these viruses. There have also been reports revealing that more than one virus can infect a host or flock either simultaneously or within a narrow period of age (22,23, 26).

The exact role each infectious agent plays in inducing enteric disease either by itself, or in combination with other agents, is not well understood. For example, it has been reported that when turkey poults were inoculated with enteric coronavirus, the result was either no clinical disease, or a mild disease was induced. Similarly, when the poults were inoculated with a strain of enteric *E. coli*, a mild to moderate enteric disease was induced. However, when this same virus was inoculated in combination with the same bacteria (i.e., *E. coli*) the resulting disease was severe with increased mortality, increased growth depression, and more severe intestinal lesions (10). Similarly, studies exploring the interactions of torovirus and bacteria in turkey poults have been reported (2). In these studies, turkey embryos were first inoculated with turkey torovirus and then at 24–36 hours postinoculation were inoculated with different bacteria. Live and inactivated (with ultraviolet radiation) *Escherichia coli* strains (one strain was isolated from the intestines of normal turkeys, and the other was the pathogenic strain O157:H7), *Streptococcus faecalis* and *Campylobacter jejuni* were used. Both live and inactivated *E. coli* and *S. faecalis* bacterial isolates did not cause lesions or disease in the intestines of dually inoculated embryos. However, when turkey torovirus inoculated embryos were inoculated with live *C. jejuni*, there was a significant increase in intestinal disease. Embryos co-inoculated with virus and UV inactivated *C. jejuni* did not cause increase in intestinal disease. Embryos inoculated with live bacteria alone did not experience any intestinal disease and were similar to embryos that were inoculated with sterile tryptose phosphate broth. It was concluded that the outcome of enteric disease during dual infection largely depends on the agents involved and that the bacteria must be live in order to produce the effect. Another example of interactions between enteric agents was illustrated in a study in which marble spleen disease virus, used as a vaccinating agent against hemorrhagic enteritis, was administered concurrently with the coccidium *Eimeria meleagrimitis* (19). This combination of agents resulted in an exacerbation of the pathogenic effect attributed to the vaccine virus. Paradoxically, results from the same study revealed that when *E. meleagrimitis* was administered concurrently with virulent HEV, the pathogenic effect attributed to the virulent HEV virus was diminished.

The observation that more than one infectious agent, noninfectious agent and / or host response may be involved in eliciting an enteric condition has prompted the use of the term multifactorial disease. In turkeys, a multifactorial enteric disease of young poults is described as poult enteritis–mortality syndrome or PEMS. PEMS has been thoroughly described in a previous edition of this textbook (6). In broiler chickens, a multifactorial enteric disease of young chicks has commonly been referred to as malabsorption syndrome. Like PEMS, numerous infectious agents have been incriminated in malabsorption syndrome of broilers (28). Both PEMS in turkeys and malabsorption syndrome in broilers still occur, are problematic for producers, are infectious and the etiology has not been determined.

Pathogenesis

The mechanism(s) by which infectious agents cause enteric disease has been reviewed by Moon (17). In his review, Moon elucidated the mechanisms that produce diarrhea. Four categories, or principal mechanisms, were identified. The first was hypermotility and was defined as the increase in intensity, frequency, or rate of intestinal peristalsis that leads to the accelerated transit of ingesta or intestinal contents through the intestines. It was also noted that decreased motility occurs in some diarrheal diseases and that alterations in motility may be an indirect effect of the enteric disease process instead of the primary cause. An alteration in intestinal permeability was identified as another mechanism of diarrhea. When alterations occur in intestinal permeability that allows the net secretion to exceed absorption, the result is increased fluid within the lumen of the intestinal tract, resulting in diarrhea. Hypersecretion was identified as the third mechanism of diarrhea. Hypersecretion was defined as the net intestinal efflux of fluid and electrolytes into the intestinal lumen that occurred despite changes in permeability, absorptive capacity, or osmotic gradients. It was further noted that mature intestinal epithelial cells, which line the distal portion of the villi, are responsible for the absorptive capacity of the intestine; whereas, the immature crypt cells are responsible for secretion. Therefore, alterations in the balance of these cells can contribute to diarrhea. For example, loss of mature cells alters the absorptive capacity and often stimulates the formation of new immature cells to replace the lost cells, thereby altering the ratio of mature to immature cells in favor of the secreting immature cells. The net result is secretory diarrhea.

Additionally, Moon reported on the effects of certain bacterial toxins that stimulate secretion of the crypt cells beyond the absorptive capacity of the mature intestinal epithelial cells that results in diarrhea. The mechanism by which bacterial toxins produce secretory diarrhea has also been observed with some enteric viruses. In the case of rotaviruses, a nonstructural viral protein (termed virotoxin) was found to be the cause of diarrhea (4, 5, 9, 11, 18).

Malabsorption was the fourth mechanism identified by Moon. Malabsorption is the process by which the absorptive capacity of the intestines is altered. This may be due to impaired absorptive capacity such as loss of mature epithelial cells. However, malabsorption is often a sequela of maldigestion because the undi-

gested feedstuffs cannot be absorbed (even though the absorptive capacity of the intestines is not impaired). In cases of malabsorption / maldigestion, nutrients within the intestinal lumen may contribute to the diarrhea by creating an osmotic effect. As is pointed out by Moon, agents that produce diarrhea are not restricted to inducing only one mechanism, but generally a combination of these various mechanisms occur.

The involvement of immune cells, and/or their products, in inducing diarrhea and enteric disease is well established. It has been reported that cytokines such as interleukins IL-1 and IL-3 (7) contribute to hypersecretion in chickens. IL-4 has been reported to influence epithelial cells and increase secretion (8). Various cytokines and biochemical mediators have been implicated in inflammatory processes leading to enteric disease and diarrhea (27). Powell suggests a neuroimmunophysiologic paradigm describing a generic model for all forms of infectious diarrhea (20). In his model, cytokines liberated from intestinal epithelial cells affect various other cells including immune cells, nerve cells, intestinal mesenchymal cells, etc. The end results include crypt hyperplasia, hypersecretion, malabsorption, alterations in permeability, etc., with the final sequela being diarrhea. It was also pointed out that this is a host defense mechanism, and although there may be points to intervene and counter the process, one must be careful not to further jeopardize the host by overzealous intervention strategies. There has been increased evidence supporting the role of the enteric nervous system (ENS) in inducing diarrhea (12). The role of the ENS has been explored with regards to bacterial and viral infections (toxins) (13,14).

In view of the aforementioned information, it was hypothesized that immune cells (or their products) may play an important role in contributing to the pathophysiology of enteric viral infections of turkeys and that the ENS may also be involved (Reynolds and Ali, personal communication). To test the hypothesis regarding immune cell involvement, turkey embryos were injected with supernatants from cultures of turkey peripheral blood lymphocytes (from normal, healthy, uninfected turkeys) that had been stimulated with conconavalin A (Con A) or with sterile media (negative control). Other embryos were injected with torovirus to serve as a positive control. Embryos injected with the Con A stimulated PBL supernatants had accumulation of fluid in the intestinal tract and decreased maltase activity that was nearly identical to the torovirus infected embryos. These studies provided evidence that products of immune cells can cause a disease in embryos that is very much like torovirus infection (1, 2, 3). Following these experiments, turkey embryos were injected with cyclophosphamide to render them immune deficient (24). The immune competent (untreated) and immune deficient (cyclophosphamide treated) embryos were inoculated (by the amniotic route) with either torovirus or with sterile media. As expected, the torovirus infected immune competent embryos developed fluid accumulation in their intestines and had decreased maltase activity as compared to noninfected control embryos. However, torovirus infected immune deficient embryos had little, if any, accumulation of fluid in their intestines, and their maltase activities were not decreased. Torovirus infection, in both the immune competent and immune deficient infected embryos, was confirmed by FA staining of the intestines (25). These studies provided further evidence that some enteric viral diseases of poultry may be a manifestation of immune cells and / or their products. To test the hypothesis regarding the role of the ENS in torovirus infections, lidocaine (a neural blocker) was injected into embyros at 48 hours and 72 hours following torovirus. It was found that the embryos that received lidocaine had a decreased secretory response (i.e., less fluid accumulation within the intestines) than those embryos that did not receive lidocaine. These results support the involvement of the ENS in diarrheic disease caused by torovirus and provide a basis for further exploring the neuroimmunophysiologic paradigm proposed by Powell for poultry. As is pointed out by Powell and others (15, 16), the relationships between infectious enteric agents, various cells, cell mediators, and body systems are extremely complex and integrated.

More recent studies have shown a role of the immune response in eliciting enteric disease in broiler chickens with malabsorption syndrome (MAS) (21, 29, 30, 31). There is evidence to indicate that a variety of immune cells, including numerous types of T cells and heterophils are involved (29, 31). Additionally, the ability to genetically select broiler chickens that are less susceptibility to MAS may be closely linked to the genetic expression of immune cells and their products (29).

Continued efforts to delineate the various factors will be needed to identify strategies for the control and remedy of diarrheic diseases. Future strategies for controlling enteric diseases may rely on drugs and biologics that may modulate the immune and neurologic component of the disease in addition to those treatment regiments directed toward specific pathogens.

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Hypoglycemia-Spiking Mortality Syndrome of Broiler Chickens

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Introduction and History

Hypoglycemia-spiking mortality syndrome (HSMS) is a disease of uncertain, but probable, infectious etiology, characterized by low morbidity and abrupt onset of high mortality (>0.5%) for at least 3 consecutive days with concurrent hypoglycemia in clinically affected birds. Seven-day-old to 14-day-old broiler chicks are usually affected (1); however, it is also frequently diagnosed in 14- to 21-day-old chicks, and the disease has been identified in commercial broilers as old as 42 days (4). Clinical signs include fine head tremors, apparent blindness, ataxia, and coma. Recovery often occurs spontaneously, but rickets and runting-stunting frequently develop in survivors.

Hypoglycemia-spiking mortality syndrome was first recognized in broiler flocks on the Delmarva Peninsula in the United States, in 1986 (3). The disease was subsequently described in 1991 (1) from 41 flocks with naturally occurring disease and 3 flocks with experimental disease. Occurrence of the disease has declined in the Delmarva area but subsequently has increased in the southeastern United States and other areas of the world.

Because the etiology is unknown and there is no specific identifying characteristic of the disease other than severe hypoglycemia, which makes only a clinical definition possible, and because young broilers experience infections with a variety of agents, the relationships among various outbreaks of HSMS remain uncertain. Two clinical forms of the disease have been identified. Type A, which was initially described, is more severe but of shorter duration than type B, a milder form occurring over a longer period, which was identified later (3). This suggests that either HSMS is one disease caused by a specific etiologic agent that occurs in different forms (possibly because of additional modifying factors) or that similar clinical diseases result from different causative agents.

Incidence and Distribution

Hypoglycemia-spiking mortality syndrome has been reported in Canada, Colombia, Europe, Great Britain, Guyana, Jamaica, Malaysia, Puerto Rico, South Africa, Thailand, Trinidad, and the United States. Although broiler chicks are most commonly affected, broiler breeder replacements and leghorns are also susceptible.

Etiology

The etiologic agent(s) of HSMS has not been identified conclusively. A serotype-12 avian adenovirus isolated from affected flocks was pathogenic for embryos and chicks but did not cause HSMS (15). Inclusion body hepatitis caused by an adenovirus was identified in a broiler flock with excess mortality and hypoglycemia (12). However, evidence for adenovirus infection in other flocks with HSMS has not been found (1, 4, 7, 8, 9).

Oral inoculations of unprocessed fecal-intestinal-saline homogenates, fecal-saline homogenates passed through 0.45-mm filters, or crude brain-phosphate buffered saline homogenates from affected chicks have been used to reproduce the disease in susceptible chicks (6, 7, 9). Hypoglycemia-spiking mortality syndrome has also been reproduced using 1) virus-like particles extracted from intestines of affected chicks and banded in a discontinuous Renograffin gradient, or 2) filtered (0.22-mm porosity filter), homogenized, dead specific-pathogen-free (SPF) embryos, which had been inoculated via the yolk sac route with the Renograffin-banded particles 72 hours earlier (8). Recently, crude pancreas homogenates have also been used to reproduce HSMS (4).

These findings indicate that at least one type of HSMS appears to be caused by an infectious, filterable agent, which may be replicated in SPF chicken embryos via the yolk sac route of inoculation (8). Attempts to replicate the agent in cell cultures have been unsuccessful (4). The agent has yet to be identified, but has no known public health significance. Arkansas strain of infectious bronchitis virus (IBV) and avian encephalomyelitis virus have been identified in some of the inocula used to reproduce HSMS experimentally (4, 8). The significance of these 2 common viral agents of chickens in the disease is currently unknown. Because particles similar to arenaviruses have been found by direct EM in droppings from affected chicks, immunohistochemistry (IHC), using polyclonal antibodies against New World and Old World arenaviruses, has been used to examine formalin-fixed tissues from naturally and experimentally infected chicks. Positive staining was found in the cytoplasm of pancreatic islets (Fig. 33.1) and acinar cells, Purkinje cells, neurons, hepatocytes, Kupffer cells, macrophages, histiocytes, and fibroblasts of infected birds but not in these cells of uninfected, matched controls (6, 7, 8). SPF embryos, inoculated via the yolk sac with Renograffin-banded virus-like particles 72 hours previously, were also IHC-positive (8). Specificity of the IHC reaction in these HSMS-infected embryos and chicks still needs to be determined. It is possible that the antibodies are cross-reacting to an epitope that is present on both arenaviruses and another unidentified agent(s). IHC (on the tissues positive with arenavirus antibodies) was negative using 2 monoclonal anti-IBV antibodies and a polyclonal antibody produced against turkey coronavirus (4).

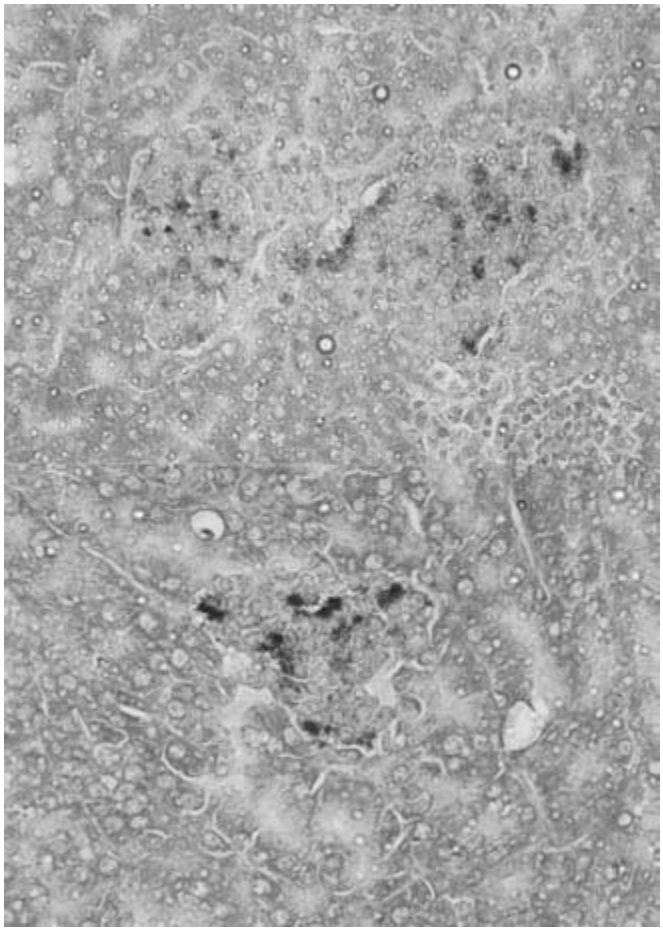


Fig. 33.1. Sections of formalin-fixed pancreas from experimentally-infected 14-day-old broiler chicks with severe HSMS, processed for immunohistochemistry (IHC). Antibody was Mouse Ascites Fluid, anti-Tacaribe (ATCC, Rockville, MD, 20852). Counterstain was Nuclear Fast Red (Sigma). Positive staining was enhanced with nickel chloride. Note the IHC positive staining (black) in pancreatic islets. The same tissues were IHC negative using negative control Mouse Ascites Fluid (ATCC, Rockville, MD 20852), and sections of pancreas from uninfected control chicks were also IHC negative.

To reproduce HSMS, 1-day-old to 2.5-day-old chicks are inoculated orally. Approximately 2 weeks later, chicks are fasted for 2–6 hours and, in some cases, sprayed with a cool (25°C) water mist to cause mild stress. Clinical signs of HSMS begin 1.5–4 hours after fasting and stressing. Plasma glucose levels are severely depressed in affected chicks; occasionally as low as 17 mg/dL. Unexposed controls remain unaffected by fasting and stressing, and plasma glucose levels remain greater than 150 mg/dL (7, 8, 9).

Hypoglycemia-spiking mortality syndrome also resulted when

birds were fed darkling beetles (*Alphitobius diaperinus*) collected from built-up litter on farms where the disease had occurred repeatedly (8). Whether beetles or other similar insects may serve as mechanical or true vectors of the agent(s) causing HSMS is still unknown.

Other factors, which some have suggested contribute to HSMS, are certain diets, especially ones with high amounts of animal by-products labile to oxidation. In the initial publication on HSMS, an all-vegetable diet was described which resulted in the highest mortality and a marked increase in susceptibility to *Escherichia coli* septicemia (3). Management errors that would lead to birds being without feed or cause them to experience other stressful events will precipitate HSMS in infected birds. Although mycotoxins or other toxic substances might be suspected in the disease because of its abrupt onset and high mortality, these substances have not been identified in affected chicks (3). Feeding cockleburs (*Xanthium* spp.) to chicks did not cause clinical disease or hypoglycemia (11).

Pathogenesis and Epidemiology

Clinical signs include huddling, trembling, blindness, loud chirping, litter eating, ataxia, prostration with outstretched legs, and coma. Rapidly growing males in good condition are often most affected (3). Chicks may or may not have diarrhea, but orange mucoid droppings are commonly found.

Gross lesions are nonspecific. Rarely, hemorrhage and necrosis are evident in the liver. There are changes consistent with mild enteritis, especially excess fluid accumulation in the lower intestines and orange mucoid enteritis in the jejunum. Similarly, microscopic changes are nonspecific and consistent with those expected from the gross lesions. Chicks with gross liver lesions have necrotic hepatocytes secondary to fibrinoid necrosis of hepatic arteries (1, 6, 8). Rarely, similar vascular changes also can be seen in the intestines and gut-associated lymphoid tissue. Rickets and severe lymphoid depletion/necrosis of the bursa of Fabricius have also been observed in affected chicks (1), although these lesions have been rare in more recent cases (4, 6, 7, 8, 9).

Experimentally, the disease seems to have a 10–12-day incubation period. At this time, chicks begin passing wet droppings containing undigested feed and they huddle together. If chicks are not fasted or stressed, this digestive disorder and the runting-stunting sequelae associated with it may be the only clinical signs seen. Surviving chicks often remain permanently stunted (7). Plasma from stunted or hypoglycemic chicks is often colorless or pale yellow compared with the deep yellow color of plasma from unaffected controls (8). Insulin-like growth factor-1 (IGF-1), a growth-related hormone (18, 20), is significantly depressed in affected chicks (7).

Acutely hypoglycemic chicks, both from the field (5) and from experimental trials (4), have significantly depressed pancreatic glucagon levels. Pancreata from these chicks are histologically normal with no evidence of cell necrosis. It has been theorized that the apparent viral infection of pancreatic islets in HSMS-affected chickens is responsible for blocking the production of pancreatic glucagon without histologically damaging the glucagon-producing islet cells (5, 7). This effect has been reported with certain arenavirus infections in other species that involve different hormone producing cells (2, 10, 16, 17, 19).

Stress and acute fasting trigger the glucagon-glycogen pathway (glycogenolysis) to maintain adequate blood glucose levels (13, 14). If chicks are deficient in glucagon and/or glycogen, they can rapidly become hypoglycemic. Chicks with HSMS are deficient in both glucagon (5) and glycogen (4, 8), which makes them extremely susceptible to development of hypoglycemia when acutely fasted and/or stressed.

Diagnosis

A high spike in a mortality curve at 7–21 days of age is suggestive but not diagnostic for HSMS. Varying numbers of chicks may be affected, or a high mortality spike may be caused by some other condition. Diagnosis of HSMS is based on the typical clinical findings and demonstrating hypoglycemia (blood glucose <150 mg/dL) in affected chicks. Plasma and/or whole blood can be used for glucose determinations; plasma levels determined with a chemical analyzer are considered to be most accurate. However, glucose test strips can be used in association with a hand held monitor (FreeStyle Blood Glucose Monitoring System, TheraSense, Inc.—Abbott Diabetes Care, Inc., Alameda, CA, 94502) as a fairly accurate screening method for determining blood glucose levels in the field or the laboratory (4). Severely affected chicks typically have blood or plasma glucose levels between 20 and 80 mg/dL. Fasting alone will not result in hypoglycemia in uninfected chicks.

Treatment, Prevention, and Control

There is no specific treatment for the disease. Supportive care based on minimizing stress due to excessive heat, cold, ammonia, poor ventilation, noise, or feed and/or water deprivation is the most important factor (4). Affected chicks should be left alone and allowed to rest as much as possible in good environmental conditions with continuous availability of feed and water. Multiple vitamins, electrolytes, and liquid vitamin E have been used successfully to reduce mortality (3, 4).

Controlled light-dark exposure programs prevent HSMS, both in the field and experimentally (8). Light-dark programs have been used successfully in the prevention of HSMS worldwide in broilers fed a wide variety of diets (4). The physiologic basis for this is attributed to melatonin release and a shift from glycogenolysis to gluconeogenesis by the birds exposed to darkness. Control of darkling beetles is important in preventing carryover of HSMS from one flock to the next. Vaccination of broiler breeder hens with an experimental formalin-inactivated autogenous vaccine produced from SPF embryos inoculated with the fourth egg passage of Renograffin-banded virus-like particles (designated The Oakwood Agent) failed to provide protection against experimental challenge of progeny from the hens (4, 7, 8). Experimental trials with "live" vaccines have not yet been attempted.

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Proventriculitis and Proventicular Dilatation of Broiler Chickens

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Introduction

Definition and Synonyms

Proventriculitis is a microscopic diagnosis characterized by the presence of inflammation in this organ. Transmissible viral proventriculitis (TVP) is an infectious, transmissible viral disease of chickens that results in enlarged, fragile proventriculi. TVP is characterized by specific microscopic changes that include glandular epithelial (oxynticopeptic) cell necrosis, ductal epithelial cell hyperplasia, and inflammation where lymphocytes predominate (2,3,11,13,19,30,33). Experiments show that TVP can be transmitted to both broiler and specific-pathogen-free leghorn chickens by a filterable agent that appears to be a non-enveloped virus (13,33). Differential diagnoses for TVP include proventriculitis caused by the ingestion of toxins such as biogenic amines, copper sulfate, or mycotoxins (4,6,17,32,36,37) or proventriculitis associated with the finding of infectious agents that include bacteria, fungi, cryptosporidia, and viruses (1,5,8,10,15,16,22, 25,28,40,42,43). In contrast, proventricular dilatation is characterized by an enlarged, but thin-walled proventriculus with no microscopic evidence of inflammation. The dilated proventriculus generally is accompanied by a small ventriculus (gizzard) and these changes are physiologic responses to diets that are low in fiber (26,34,38).

Economic Significance

Chickens affected by TVP may cost more to produce than unaffected chickens due to poor food digestion, poor feed conversion, and stunted growth (7,11,15,19,24,33). Rupture of the proventriculus or gastric isthmus during carcass evisceration results in carcass contamination and significant economic loss (13,15,23, 28,29,31).

Public Health Significance

When chickens with either proventriculitis or proventricular dilatation are slaughtered and processed there may be contamination of the carcass and processing equipment with ingesta or intestinal contents (2,13,15,31). Increased reprocessing and condemnation costs are incurred in an effort to remove potential human pathogens (23,39).

History

Proventricular dilatation (enlargement of the proventriculus without inflammation) was first described in chicks that were fed a purified ration (26), and later described in commercially raised broiler chickens that were fed a ration low in fiber. In these chickens the proventriculi were dilated and thin-walled and gizzard musculature was underdeveloped (34). Later, it was discovered that proventricular enlargement, with inflammation, could also be induced in chickens that were fed toxins such as biogenic amines (36), copper sulfate (32), and mycotoxins (6). A transmissible proventriculitis that produced a runting syndrome affecting broilers was first identified in Holland (19). A similar syndrome associated with stunted growth and proventriculitis was then described in the United States of America (27) and later recognized in Australian broiler chickens (33). Other features of this illness included whole-body pallor, fragile proventriculi, a dilated and weakened gastric isthmus, poor feed conversion ratios, and the passage of undigested or poorly digested feed in the feces (7,9,11,13,15,24,31,33). In an examination of field cases of proventriculitis in the USA, Goodwin *et al.* (11) identified 60–70 nm adenovirus-like viruses in the nuclei of degenerating alveolar epithelial cells, leading to the designation of this condition as transmissible viral proventriculitis (12).

Incidence and Distribution

Definitive prevalence data regarding the global incidence and distribution of proventriculitis and proventricular dilatation are not available. However, with regard to TVP, a single study in the USA identified the typical microscopic lesions of this disease in nearly half of the broiler chicken proventriculi examined microscopically (11). A similar proventriculitis had been previously identified in Holland (19,20,21) and has been more recently described in Australia (33).

Etiology

Classification

The cause of TVP is a virus that has not been conclusively characterized. Some investigators have identified a distinctive adenovirus-like virus in naturally occurring (11,33) and experimentally induced TVP (13). PCR examination of nucleic acids extracted from this virus using primers specific for group I, II, and III avian adenoviruses did not result in an amplified product (13).

Morphology

In some thin sections of proventriculi affected by TVP, distinctly hexagonal (icosahedral), nonenveloped virions (average particle size 65–70 nm) have been identified in the nuclei of glandular epithelial cells (11,13). Virions of a similar size and shape also have been detected in proventricular glandular epithelial cells of some Australian chickens affected by TVP (33) and similar, but larger (100 nm) hexagonal virions have been noted in experimentally infected chickens (15). In glandular epithelial cells with fragmented nuclei, virions were present in the cytoplasm associated with unbound condensed chromatin (11).

Laboratory Host Systems

Chickens commonly have been utilized as laboratory host systems for TVP. The disease has been experimentally reproduced by inoculation of SPF and commercial broiler chickens with filtered or nonfiltered homogenates of proventriculi collected from TVP-affected chickens (2,13,15,29,30,31,33). An adenovirus-like virus associated with TVP has been isolated from the proventriculi of TVP-affected chickens by sequential passage in SPF embryonated chicken eggs inoculated via the amnionic route, but attempts at culturing this virus on confluent cell culture monolayers have not been successful (13).

Pathogenesis and Epidemiology

Natural and Experimental Hosts

Naturally occurring TVP has only been reported in commercially-raised broilers; however, the disease is transmissible to both broilers and SPF leghorns (2,13,15,29,30,31,33).

Age of Host Commonly Affected

TVP is primarily a disease of young (3–8 week old) broiler chickens. The disease has been reported to occur most commonly in 4–5 week old chickens (2); however, in some experimental studies, the highest lesion prevalence was observed 4 weeks after inoculation (33). In two separate studies, broiler chicks inoculated at 1 day of age and SPF chickens inoculated when they were 2 weeks old both developed proventricular enlargement between 1 and 2 weeks post-inoculation. The source material for these experimental studies were proventriculi obtained from 2- to 4-week, TVP-affected commercial broiler chickens (13,30).

Transmission

Routes of natural infection of TVP are not known; however, chickens can be infected experimentally by oral gavage or oculonasal instillation of either crude or filtered (0.2 μm) proventricular homogenates (2,3,13,15,19,29,30,31,33). The disease has also been experimentally reproduced using filtered proventricular homogenates that had been treated with chloroform to eliminate enveloped viruses (13,33).

Incubation Period

The incubation period of naturally occurring TVP has not been defined although grossly enlarged proventriculi have been reported in 3 week old broiler chickens with naturally-occurring TVP, but not in 1–2 week old broiler chickens (13). Experimental studies involving day-old chicks suggest that homogenates of proventriculi from these chicks were infectious 2 weeks after in-

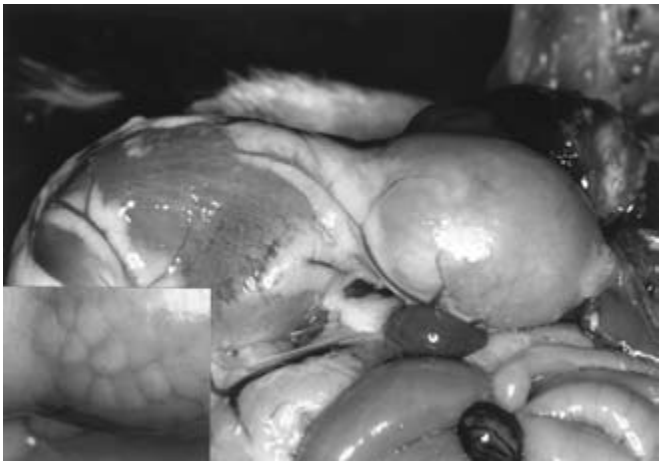


Fig. 33.2. Proventriculus from a virus-infected broiler chick is enlarged and mottled gray-white-yellow. Close examination of the serosal surface reveals numerous polygonal pale foci that represent enlarged individual glands (inset).



Fig. 33.3. The proventricular mucosa of a virus-infected chick appears thickened and rugose. Papillary orifices are not distinct.

oculation (33). In some studies proventricular enlargement occurred within 10 days post-inoculation (13).

Clinical Signs

The predominant signs in chicks with TVP include stunted growth, pallor, unthriftiness, and passage of undigested or poorly digested feed in their feces (11). Naturally occurring cases are associated with poor flock production performance data (9,33). No clinical signs are apparent in chickens with proventricular dilatation (38).

Morbidity and Mortality

There is no increased mortality in TVP affected flocks (33), but an estimated 1% may require reprocessing due to carcass contamination at slaughter (39).

Pathology

Gross Lesions

Chicks with naturally-occurring or experimentally-induced TVP may be significantly smaller than their uninfected counterparts (7,15,33). At necropsy, the isthmus between the proventriculus and ventriculus is widened and the proventriculus is enlarged with thickened walls that are mottled gray-white-yellow (Fig. 33.2). The lobular pattern is often pronounced with each pale polygonal focus representing an individual gland (11,31,33). Some glands are distended and a viscous white material can be expressed by gentle digital pressure. The luminal mucosa may appear thickened and rugose and papillary orifices may be indistinct (Fig. 33.3).

In chickens fed excessive levels of copper, proventriculi are enlarged, walls are thick, and the mucosal lining is flattened and stippled brown-black (17). Histamine produces enlarged, flaccid proventriculi with mucosal erosions (36). Similar mucosal erosions are present in chickens exposed to some mycotoxins (6). Proventriculi from chickens that have been fed low fiber diets are dilated and thin-walled and gizzard musculature is poorly developed (34,38).

Microscopic Lesions

Light Microscopy. Microscopic lesions in TVP-affected chickens change during the course of infection. At 5 to 7 days postinfection (DPI) experimentally infected chicks exhibit acute necrosis of the glandular epithelial cells that produce pepsinogen and hydrochloric acid. The cytoplasm of these cells is amorphous, granular, or vacuolated and there are pyknotic or fragmented nuclei. In the nuclei of a few attached or sloughed glandular epithelial cells, nuclear chromatin is marginated and central areas are pale-staining, but there are no distinct hyaline or basophilic inclusion bodies. This necrosis is accompanied by accumulations of lymphocytes, macrophages, and fewer plasma cells in the adjacent connective tissue stroma (tunica propria). Collecting ducts (secondary ducts) are dilated and filled with necrotic cells (Fig. 33.4) and there may be hyperplasia of the gut-associated lymphoid tissue (GALT) that normally occurs in the proventricular glands. During the transitional stage of infection (14 DPI) there is regeneration of the glandular epithelium by a cuboidal to low columnar duct-like epithelium. In later stages, glandular epithelial cells are partially replaced by hyperplastic ductal epithelium (Fig. 33.5), and there is an accompanying lymphocytic proventri-

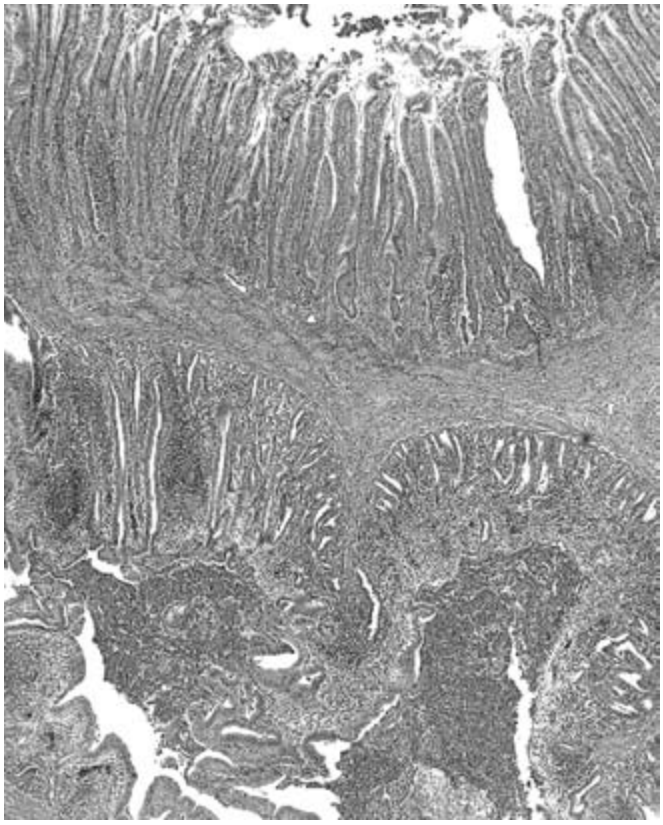


Fig. 33.4. In acute naturally-occurring proventriculitis, collecting ducts (secondary ducts) are widely dilated by accumulations of necrotic alveolar epithelial cells. H&E 40×

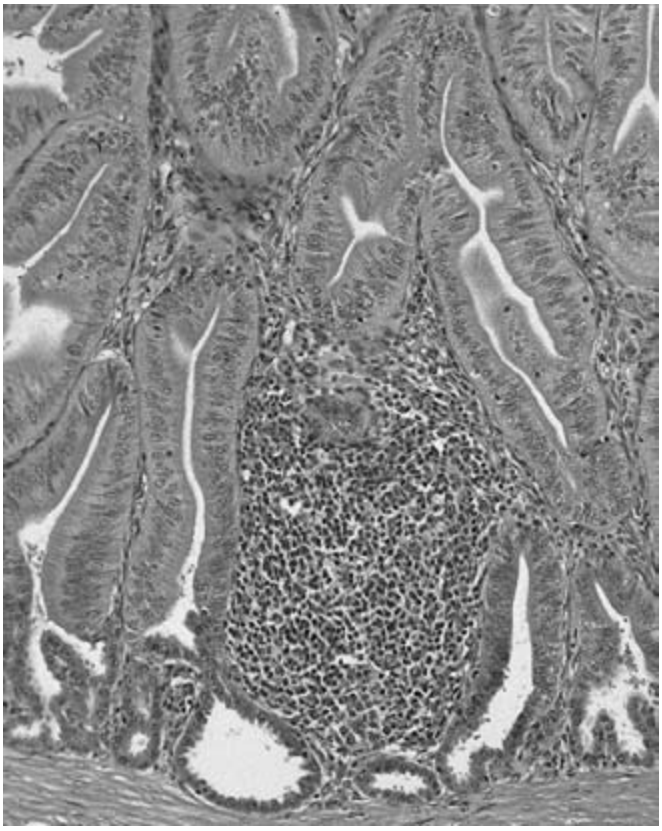


Fig. 33.5. In chronic lesions, there is marked hyperplasia of duct-like epithelium that extensively replaces alveolar epithelial cells with accompanying interstitial aggregates of lymphocytes and plasma cells. H&E 200×

culitis with GALT hyperplasia and occasional B cell aggregates that form germinal centers (13,30,31). In naturally-occurring and experimentally-induced disease, there may be destruction of up to 80% of the glandular epithelial cells.

Proventriculitis in chickens can occur secondary to ingestion of excessive levels of histamine with lesions consisting predominantly of mucosal erosion and edema (36). Dilated proventriculi with erosion or ulceration of the luminal mucosa coupled with submucosal inflammation are seen in chickens with experimental cyclopiazonic acid intoxication (6).

There are no light microscopic lesions in physiologic proventricular dilatation.

Electron Microscopy

In some chickens with naturally- or experimentally-produced TVP there are intranuclear hexagonal (icosahedral) viral particles (Fig. 33.6) that have been reported to range in size from 60–70 nm (11,13). Viral particles in intact nuclei tend to be larger than those found in the cytoplasm of cells with fragmented nuclei (11). Adenovirus-like viruses associated with TVP (Fig. 33.7) appear to be approximately 70 nm in diameter; these viruses resemble adenoviruses, but lack characteristic surface structures and electron-dense cores (13).

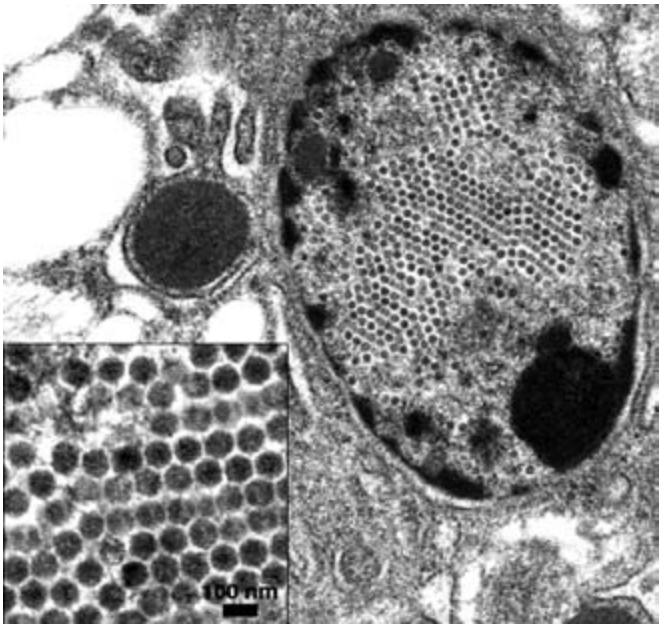


Fig. 33.6. There are intranuclear, crystalline arrays of adenovirus-like viruses in the nucleus of a proventricular epithelial cell from an experimentally infected chick. Icosahedral, approximately 70 nm virions are present in crystalline arrays (inset). (*Avian Diseases*)

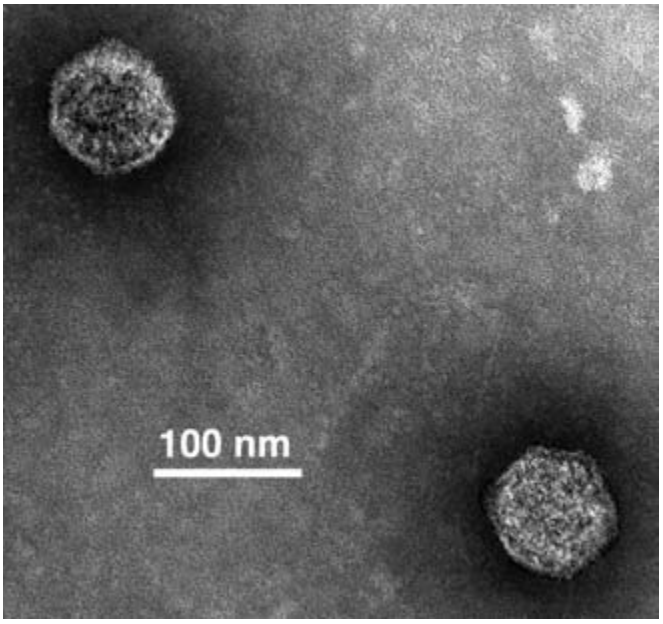


Fig. 33.7. Negatively stained viral particles from an experimentally infected chick are icosahedral and are approximately 70 nm in diameter. (*Avian Diseases*)

Immunity

Nothing is known about immunity to TVP.

Diagnosis

Isolation and Identification of Agent

Studies of TVP that used 0.2 μm filtered and chloroform-treated proventricular homogenates to reproduce the disease suggest that the etiologic agent is a non-enveloped virus (13,33). In some experiments that successfully reproduced the proventriculitis, there was no evidence of either reovirus or Newcastle disease virus (31,33). While some trials have indicated the variable presence of infectious bursal disease virus or infectious bronchitis virus (2,15,31), other studies have not detected either one (13,28,29) or both (33) of these agents. Several studies have indicated the presence of avian adenoviruses (31,33) or adenovirus-like virus particles (11,13,15).

The intranuclear 60–70 nm hexagonal (icosahedral) adenovirus-like virus identified in the proventricular epithelium of chickens naturally (11,33) and experimentally (13) infected with TVP has been successfully propagated by serial passage in embryonated eggs inoculated by the amnionic route (13).

Serology

There is no serologic test for TVP.

Differential Diagnosis

A tentative diagnosis of TVP is made on the basis of the typical microscopic lesions in the proventriculus. TVP must be differentiated from a broad spectrum of offending agents reported to cause proventriculitis or proventricular enlargement in chickens. These include copper sulfate ingestion (3,32), dietary biogenic amines and mycotoxins (4,6,10,36,37), bacteria (10,15), fungi (10,35,40), reovirus (18,22,27), adenovirus (19,22), infectious bronchitis virus (43), tumor-inducing viruses (1,16,25,41,42), *Cryptosporidium* sp. (8), and idiopathic proliferations of cells interpreted to represent histiocytes (14). A diagnosis of proventricular dilatation relies on observing a dilated, thin-walled proventriculus without microscopic evidence of inflammation (34,38).

Treatment

There are no specific treatments for proventricular dilatation or TVP.

Prevention and Control

There are no specific prevention or control measures for TVP.

Proventricular dilatation may be prevented by ensuring proper ration formulation, specifically by providing adequate dietary fiber.

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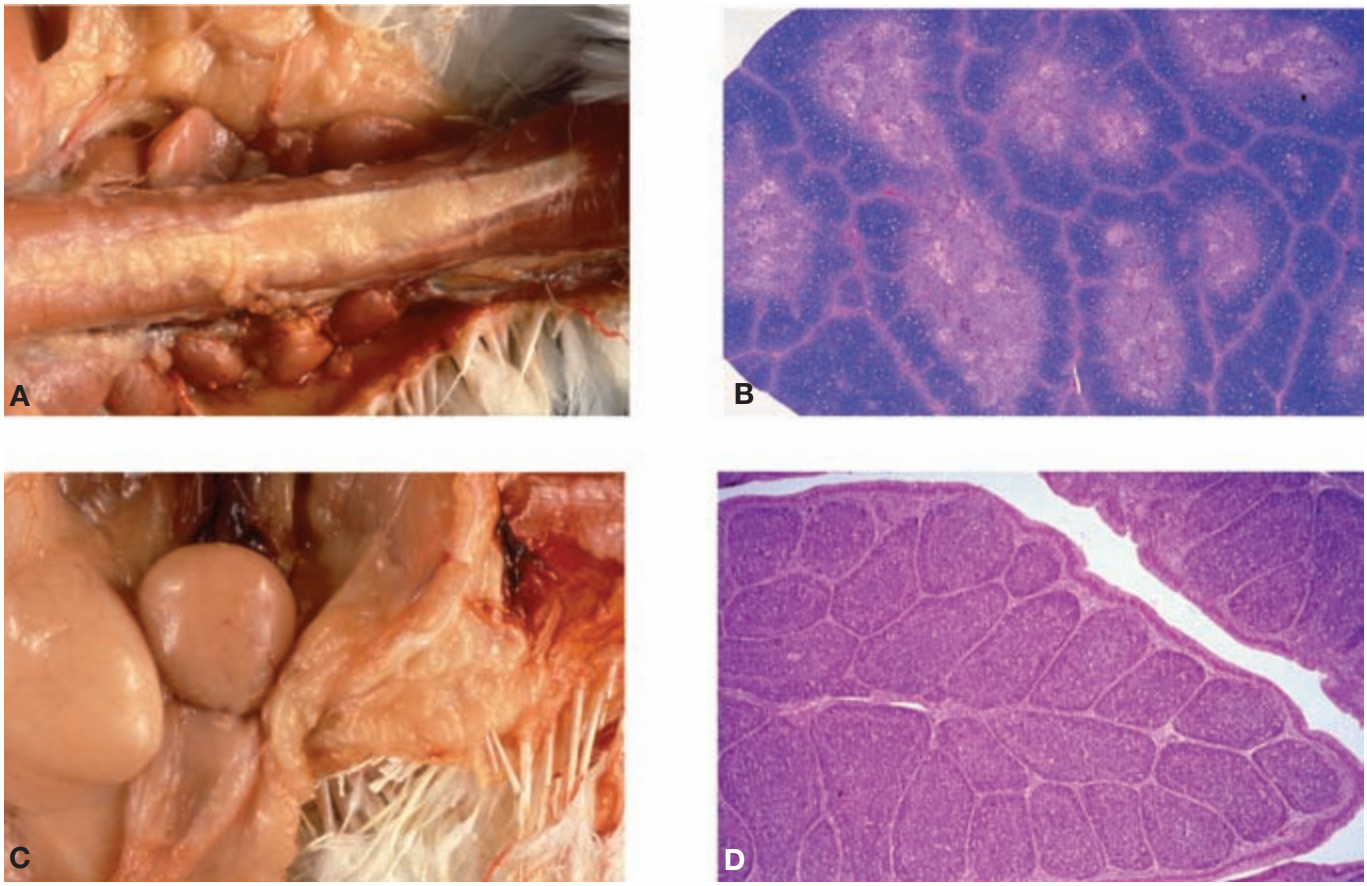
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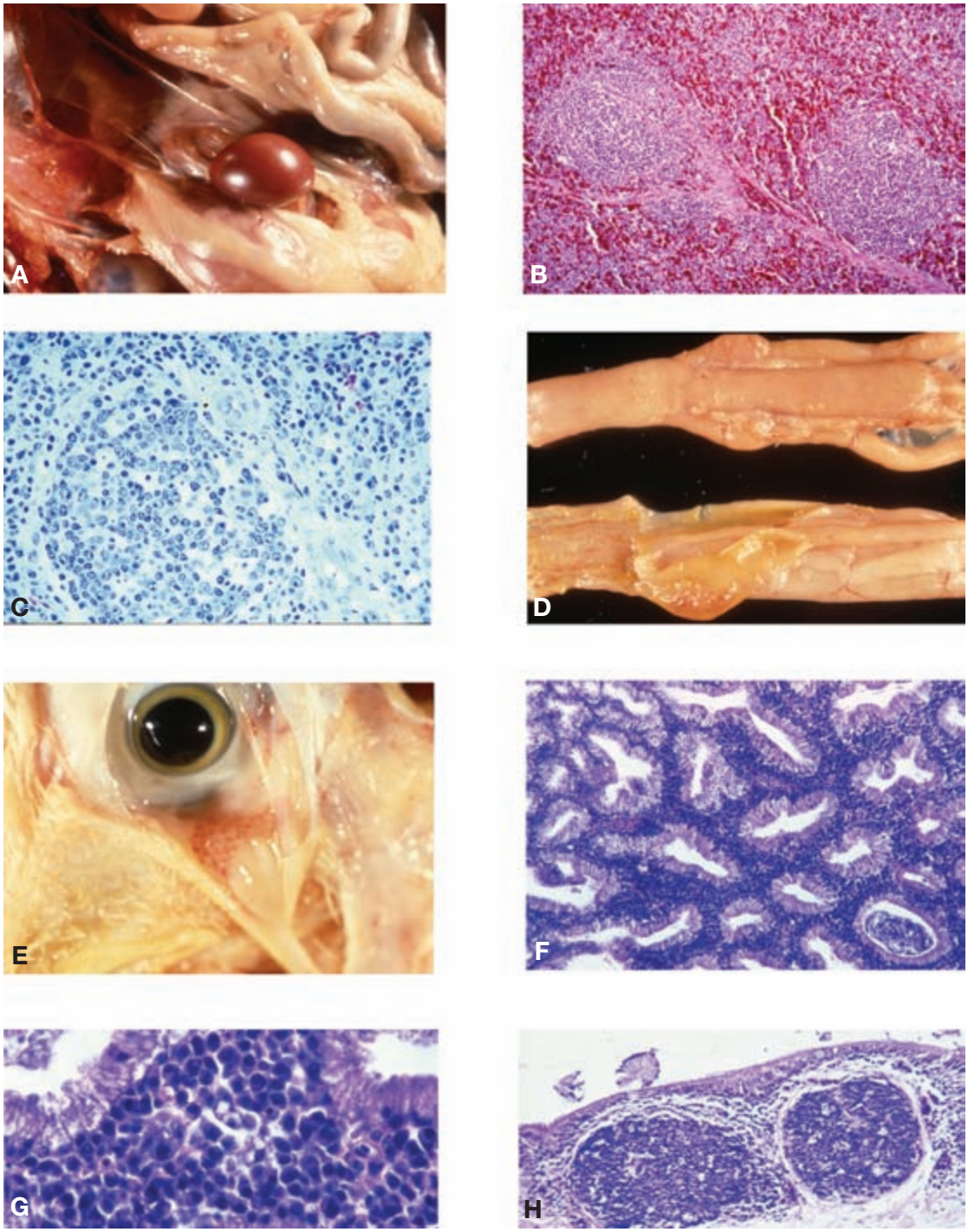
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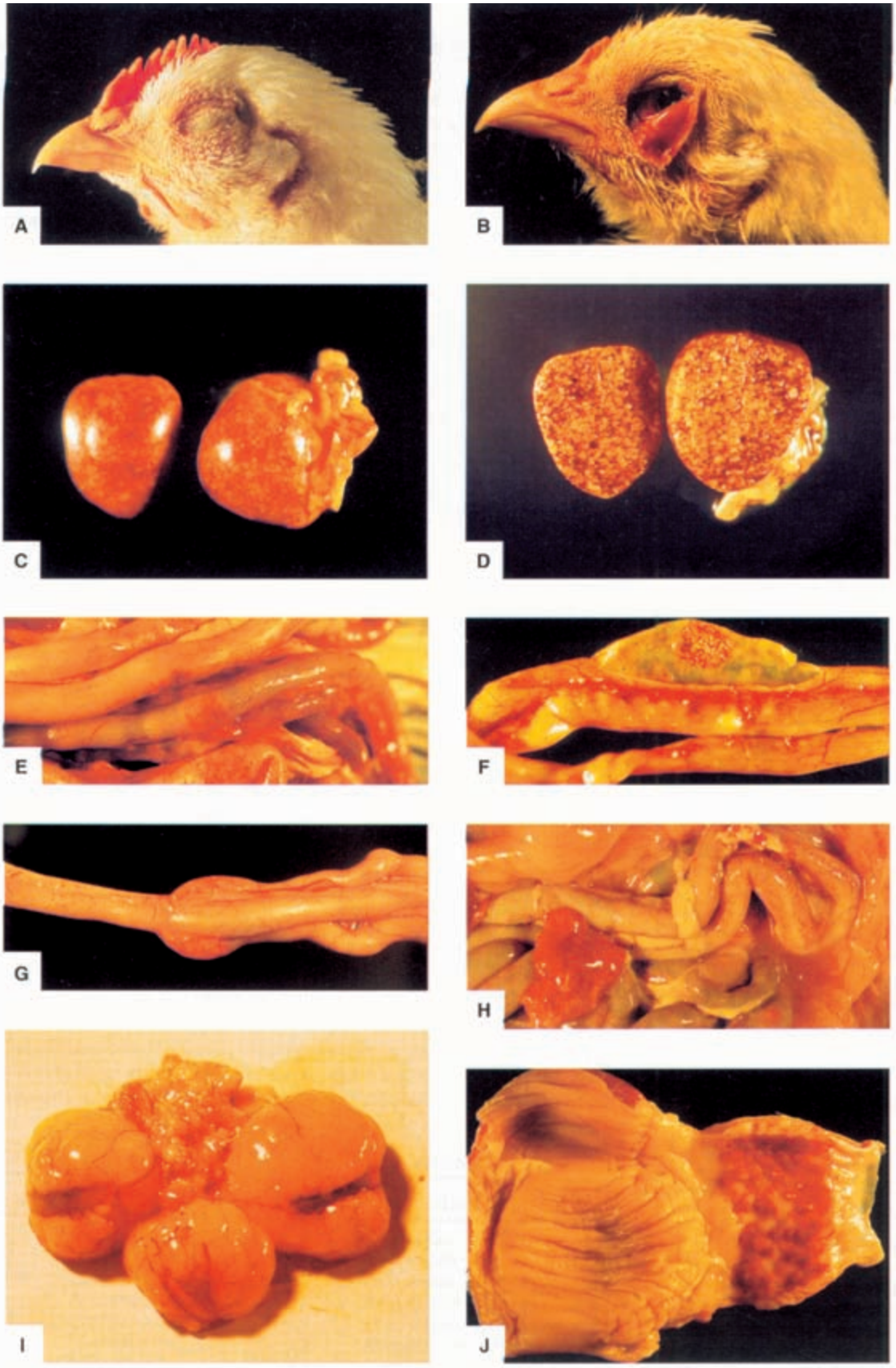
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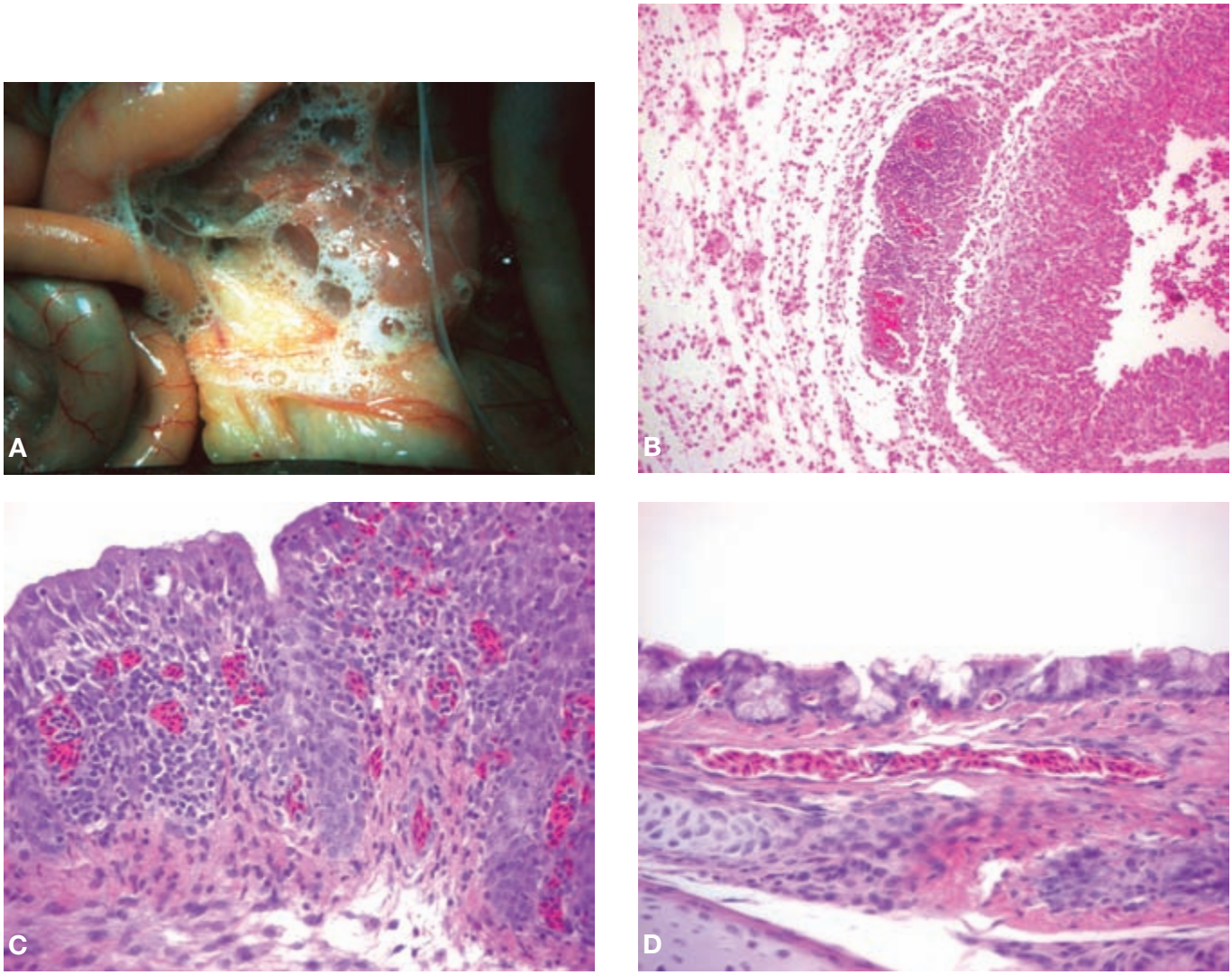
2.1. A. Multiple lobes of the thymus lie on each side of the trachea. B. The bursa of Fabricius is the sac-like structure extending from the end of the intestine. C. The multilobular histologic structure of the thymus is evident; each lobule is composed of the dark-staining cortex and the pale medulla. D. Bursal lymphoid follicles are separated by thin connective tissue septae. (From Fletcher, O. J., and H. J. Barnes. 1998. Lymphoid organs and their anatomical distribution. In J. M. Sharma (section ed.). Avian immunology. In P. P. Partoret, P. Griebel, H. Bazin, and A. Govaerts (eds.). *Handbook of Veterinary Immunology.* Academic Press. With permission.)



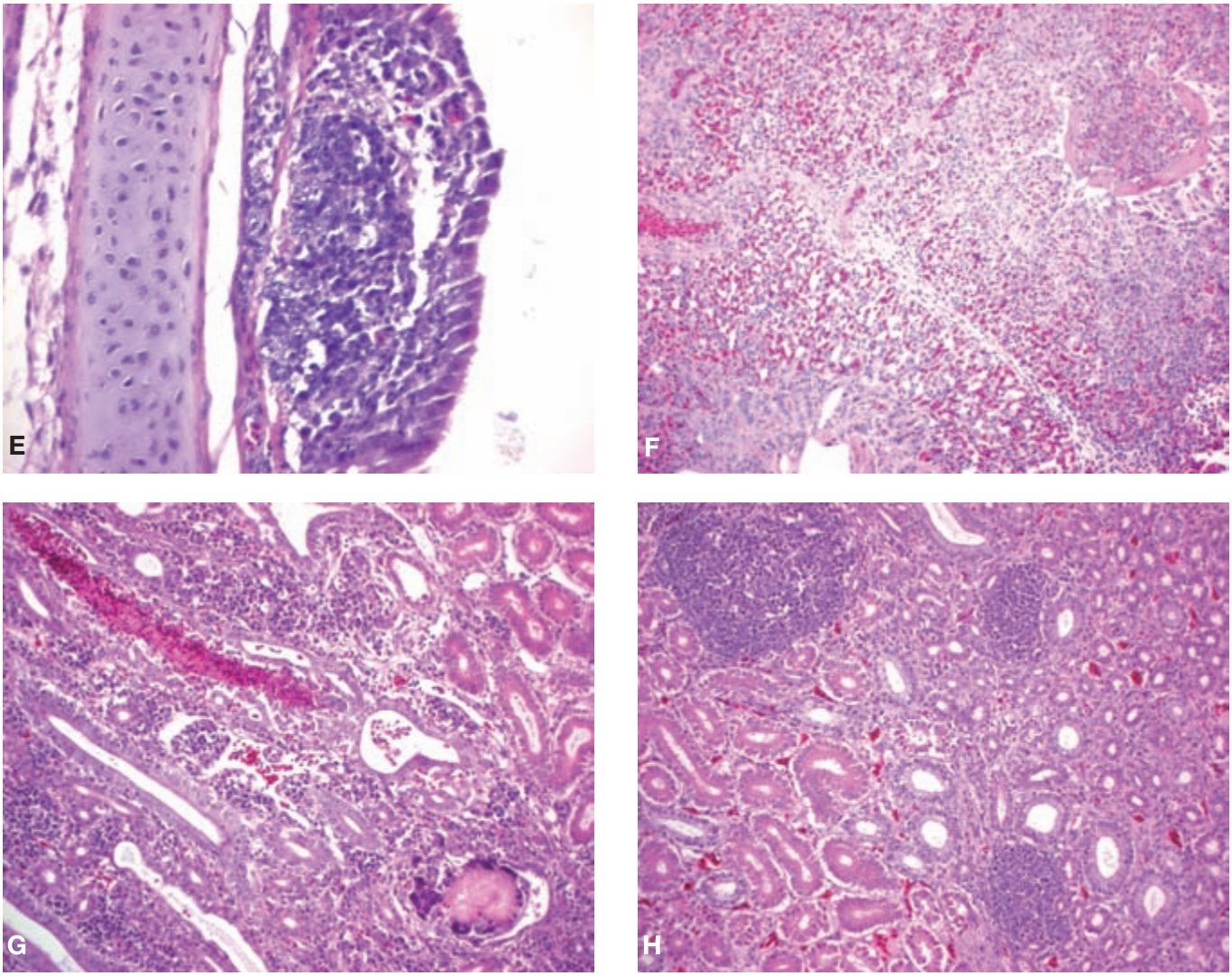
2.2. A. The spleen is the oval organ in the central area of the photograph. B. Periarterial lymphoid sheaths are located in the white pulp of the spleen. C. A bursa-dependent lymphoid follicle is located adjacent to a small artery and surrounded by thymus-dependent lymphoid cells. D. Cecal tonsils, unopened (top) and opened (bottom). E. Small nodules in the conjunctiva and the conjunctival-associated lymphoid tissue (CALT). F. The Harderian gland contains lymphoid cells in the connective tissue between the glands. G. Plasma cells are the predominant cell population in the Harderian gland. H. Nodular deposits of lymphoid tissue are located in the mucosa of the trachea. (From Fletcher, O. J., and H. J. Barnes. 1998. Lymphoid organs and their anatomical distribution. In J. M. Sharma (section ed.). Avian immunology. In P. P. Partoret, P. Griebel, H. Bazin, and A. Govaerts (eds.). *Handbook of Veterinary Immunology*. Academic Press. With permission.)



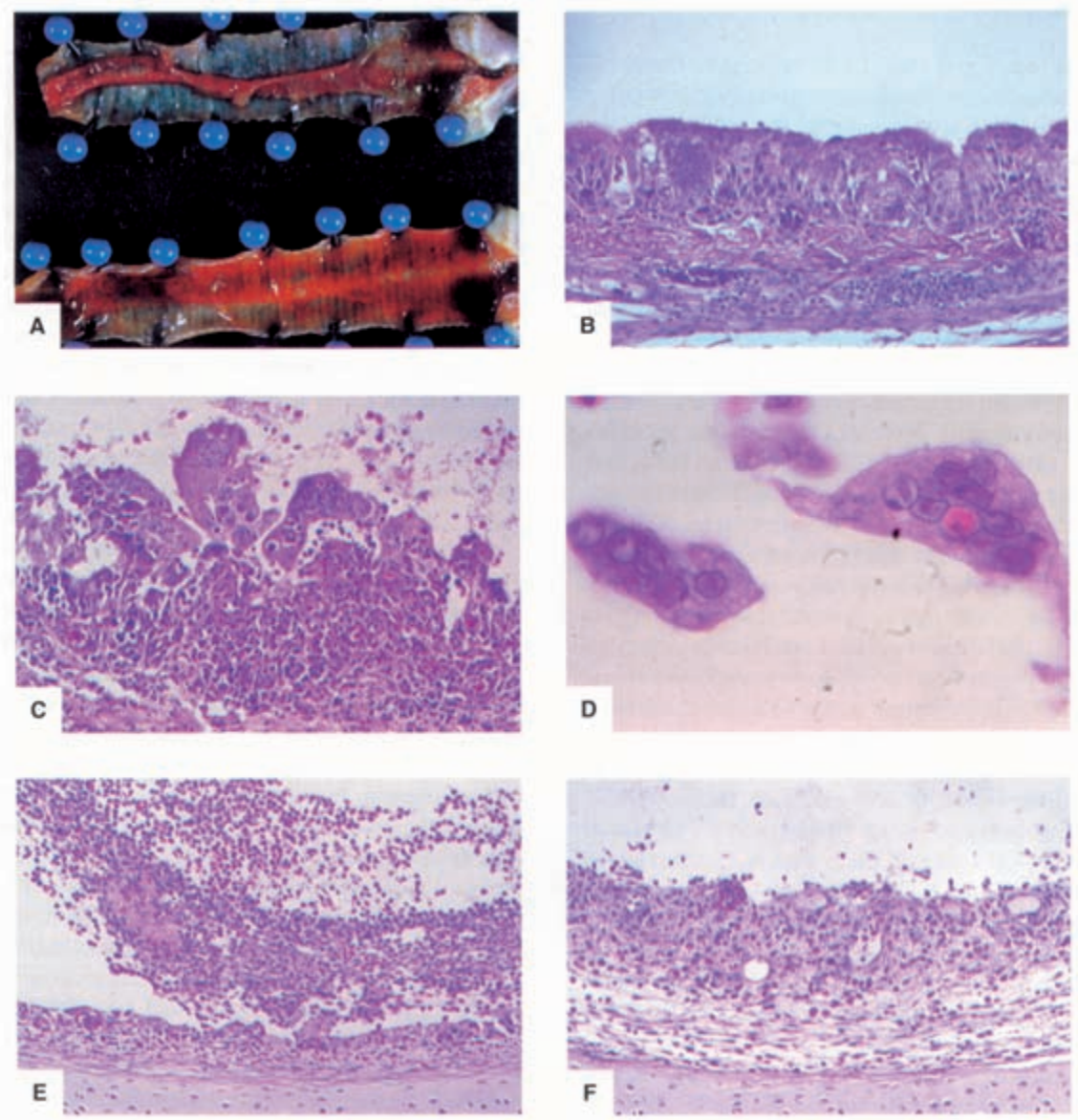
3.2. Gross lesions of velogenic viscerotropic Newcastle disease in susceptible chickens inoculated by the eyedrop route. A. Facial edema. B. Hemorrhage, congestion, and conjunctivitis in reflected eyelid. C–D. Splenic necrosis on the capsular surface (C) and cut surface (D). E–F. Necrosis and hemorrhage in intestinal lymphoid aggregates evident from the serosal surface (E) and mucosal surface (F). G. Enlarged and necrotic cecal tonsils. H. Peritonitis with fibrin deposition. I. Ovarian follicles with hemorrhagic stigmata. J. Hemorrhage in the mucosa of the proventriculus. (A–I, King and Swayne; J, Beard)



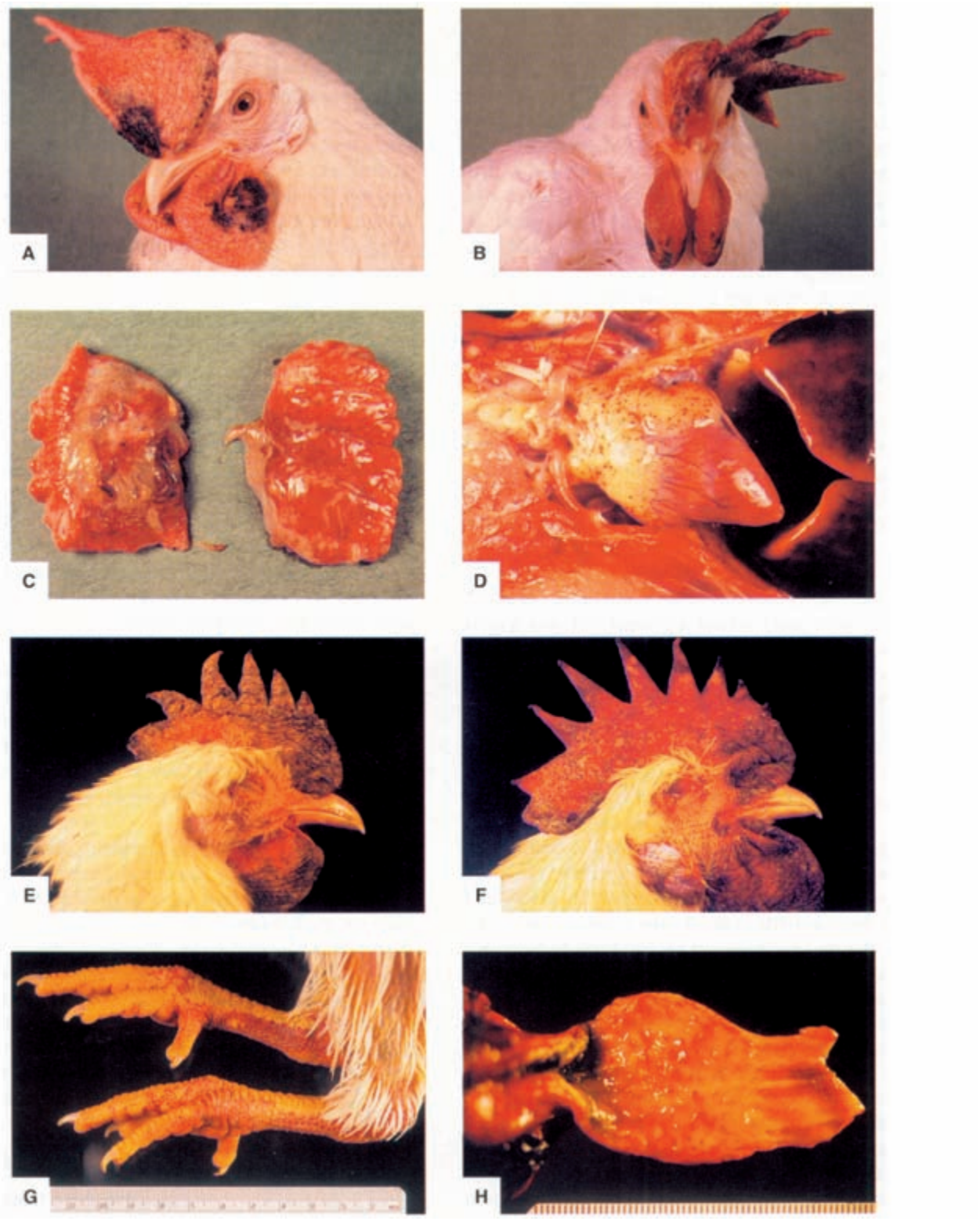
4.6. A. Foamy airsacculitis associated with an early field infection with the Delaware 072 strain of IBV (University of Delaware; Ed Odor). B. Airsacculitis from a severe IBV field vaccine reaction in a four-day-old broiler. Inflammation related thickening of the air sac wall with heterophils and macrophages present in the air sac lumen. Peripheral to the air sac wall is soft tissue inflammatory edema to include lymphocytic perivascular cuffing around congested blood vessels. C. Viral trachitis related to an experimental infection with the Arkansas Strain of IBV at post-innoculation day 3. Visible are cilial loss, partial mucous gland depletion, and mucosal epithelial degenerative changes to include rare heterophil infiltration, mucosal epithelial hyperplasia, congestion, lymphocytic perivascular cuffing, and submucosal edema. D. Normal trachea from sham innoculated negative control at post-innoculation day 3 for comparison with C. Cilia and mucous glands are prominent, and there is a lack of inflammation-based mucosal thickening.



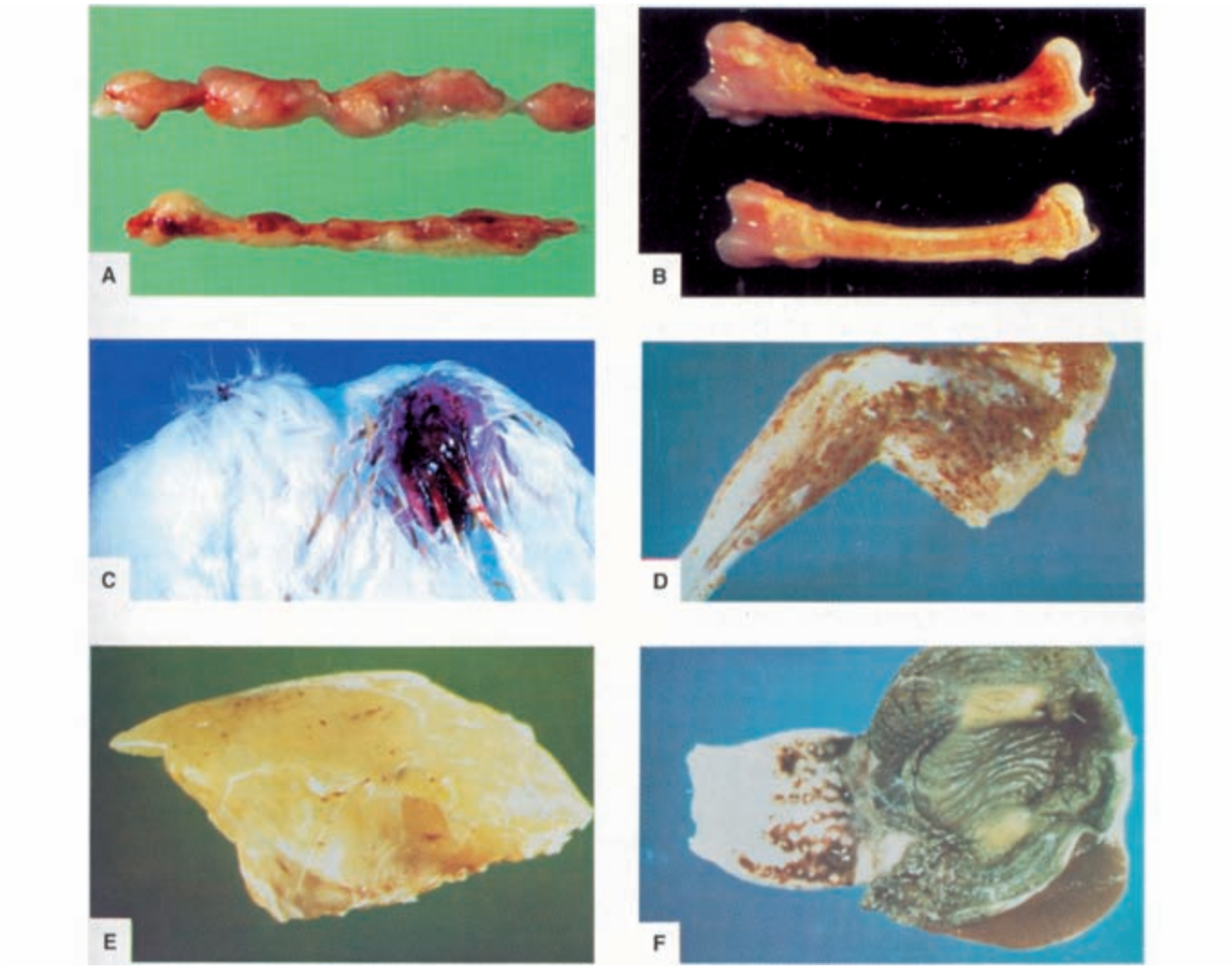
4.6 *(continued)* E. Trachea from an experimental IBV infection at post-innoculation day 14. There is a lack of acute inflammation. Cilia are apparent; the mucosa contains a developing germinal center; and lymphocytes are present in the submucosa. F. Broncho-pneumonia related to an experimental infection with the Arkansas strain of IBV at post-innoculation day 6. A heterophilicbronchopneumonia involves a parabronchus resulting in the filling of the parabronchial lumen with heterophils and heterophilic infiltration of associated exchange tissue. A portion of a normal parabronchus is adjacent to the parabronchus with the bronchopneumonia. G. Tubulointerstitial nephritis of a medullary cone at post-innoculation day 10 following exposure to the nephrogenic PA/Wolgemuth/98 strain of IBV. Degenerated-necrotic misshapened, often dilated, tubules containing urate, heterophilic, and degenerated epithelial casts are accompanied by an interstitium expanded by mostly plasma cells, lymphocytes, and a few heterophils. H. Germinal centers in and at the margin of a medullary cone are an indication of an area of near complete healing at post-innoculation day 10 following exposure to the nephrogenic PA/Wolgemuth/98 strain of IBV (University of Delaware; Conrad R. Pope, Brian S. Ladman, and Jack Gelb, Jr.).



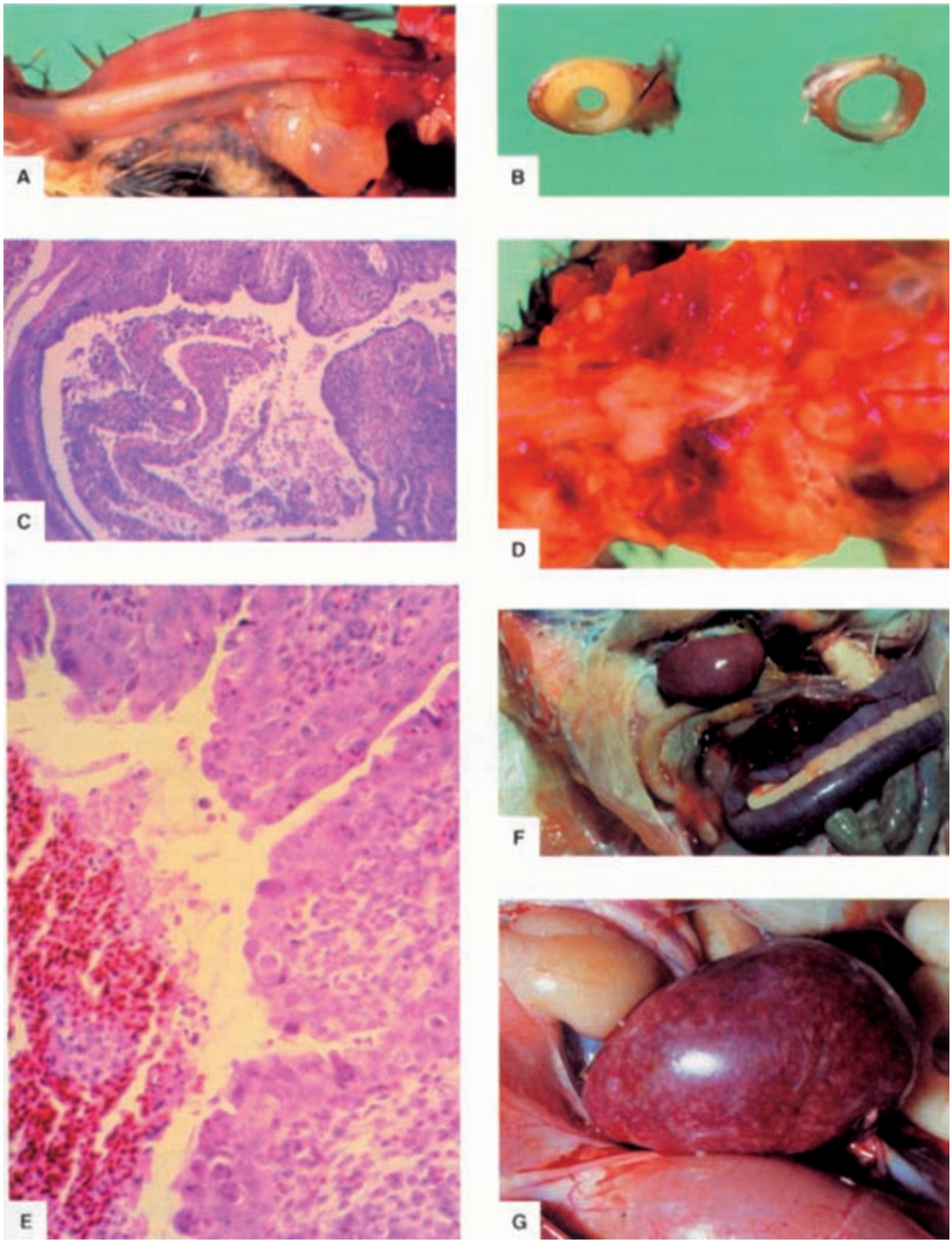
5.5. *A.* Fibrinohemorrhagic tracheitis in chickens with laryngotracheitis. (Munger). *B-F.* Microscopic tracheal lesions of laryngotracheitis. *B.* Early laryngotracheitis lesions in the trachea. Mucosa is slightly thickened. There is a mild infiltration of lymphocytes in the mucosa and submucosa especially around vessels. A large multinucleated syncytial cell has developed in the mucosa. *C.* Numerous syncytial cells have separated from the mucosa, which is now heavily infiltrated by lymphocytes. Presence of syncytial cells in the tracheal lumen is characteristic of laryngotracheitis. *D.* High magnification of sloughed syncytial cells showing numerous intranuclear inclusions. *E.* Later in the disease, epithelium, blood, and inflammatory exudate form a membrane that separates and sloughs into the lumen. This may occlude the trachea and cause death from asphyxiation or serve as a medium for bacterial proliferation. Expelling the exudate by coughing is a classic clinical sign of laryngotracheitis. *F.* The amount of epithelium that survives depends on virulence of the strain. This bird was infected with the Illinois strain, which is highly virulent. As a result, there has been complete loss of epithelium, leaving lamina propria exposed.



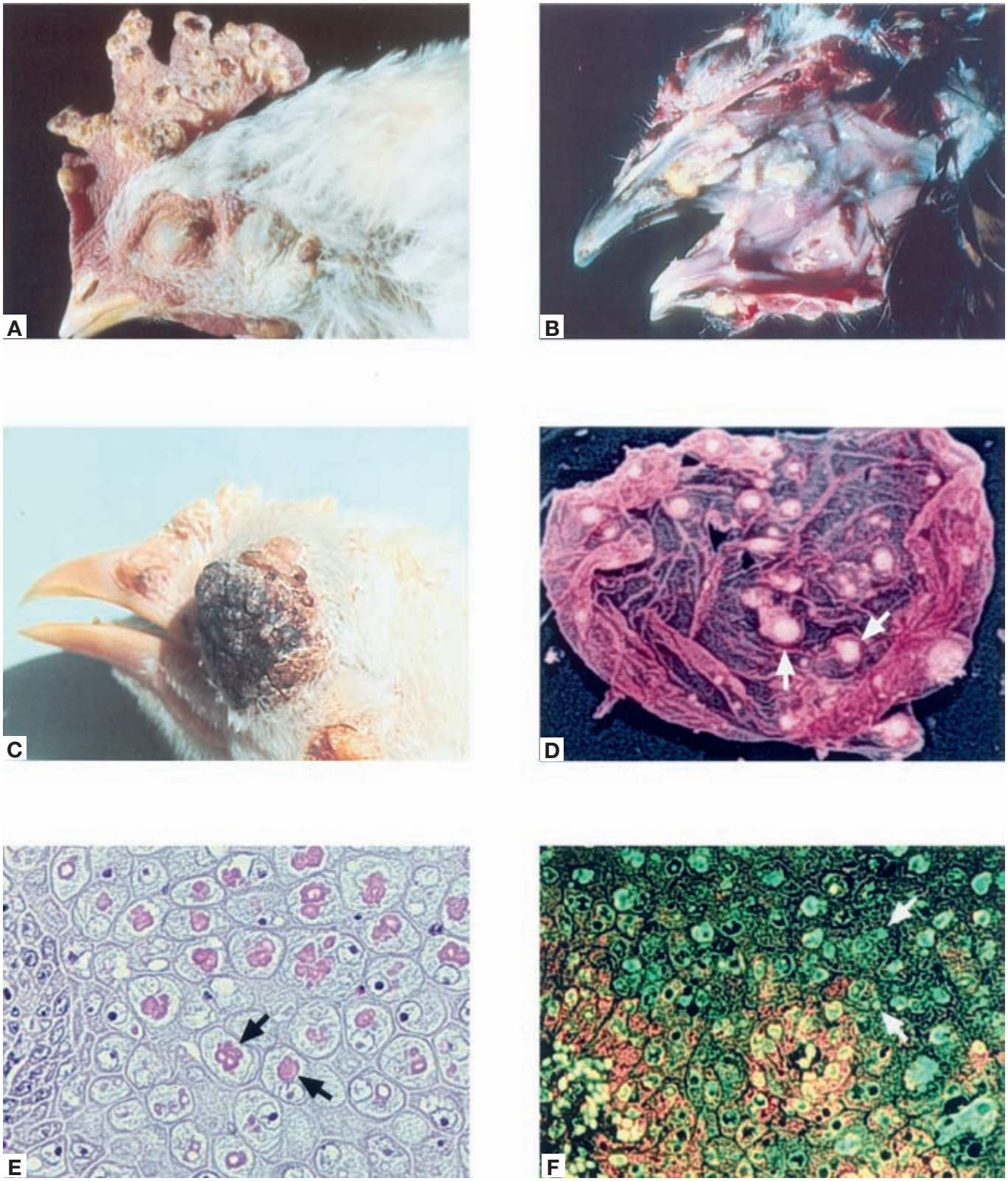
6.2 Gross lesions associated with experimental infection of white leghorn (WL) or White Rock (WR) chickens with highly pathogenic (HP) avian influenza viruses. A–D. Lesions in adult WL chickens, 47–59 weeks of age, exposed to HP A/chicken/NJ/12508/86 (H5N2) derivative influenza virus by the intranasal/intratracheal routes. A. Multifocal necrosis and hemorrhage of comb and wattles 7 days post infection (DPI). (USDA—Brugh) B. Severe edema, necrosis, and hemorrhage of comb and wattles, 7 DPI. (USDA—Brugh) C. Bilateral ventral medial pneumonia with edema, 3 DPI. (USDA—Brugh) D. Petechial hemorrhages in epicardial fat, 4 DPI. (USDA—Brugh) E–H. Intranasal (IN) or intravenous (IV) exposure of immature chickens to HP A/chicken/Queretaro/14588-660/95 (H5N2) virus stock. E. Severe necrosis of comb and wattles, 12-week-old WL, IN exposure, 4 DPI. (USDA—Swayne) F. Severe edema and necrosis of comb and wattles 12-week-old WL, IN exposure, 4 DPI. (USDA—Swayne) G. Severe subcutaneous hemorrhages of leg shanks, 4-week-old WR , IV exposure, 4 DPI. (USDA—Swayne) H. Petechial hemorrhages around the ducts of the proventricular glandular region, 16-week-old WL, IN exposure, 4 DPL. (USDA—Swayne).



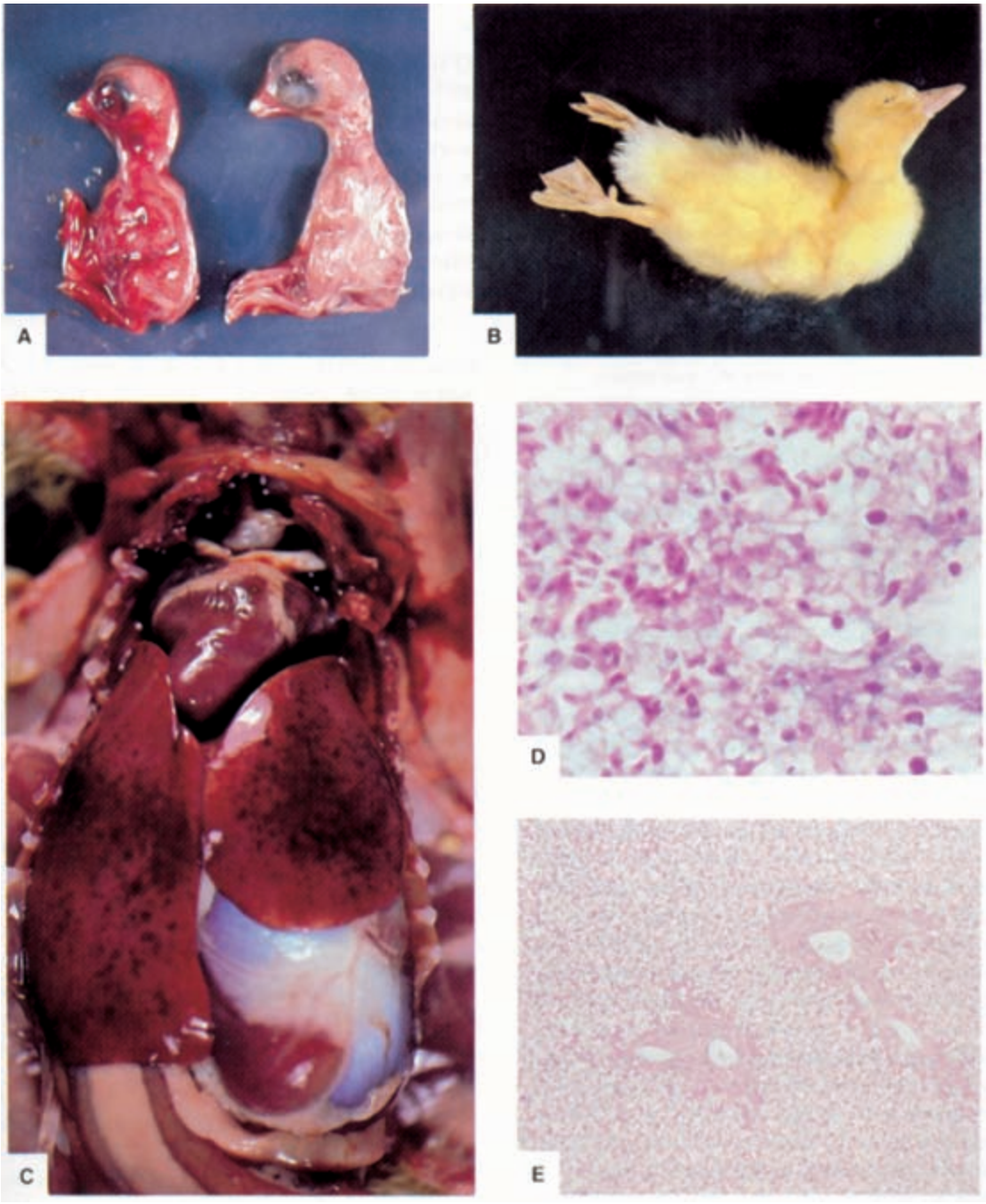
8.2. Lesions in chickens associated with chicken infectious anemia and hemorrhagic anemia disease. A. Control thymus (top) and thymus with chicken infectious anemia virus (CIAV)-induced atrophy (bottom), 14 days postinoculation with the CIA-1 strain of CIAV. (*Lucio and Shivaprasad*). B. Femur with normal dark red bone marrow (top) and femur with pale aplastic bone marrow (bottom), 14 days postinoculation with the CIA-1 strain of CIAV. (*Lucio and Shivaprasad*). C. Gangrenous dermatitis (blue wing disease). (*Shivaprasad*). D. Hemorrhages in thigh and leg muscles. (*Peckham*). E. Hemorrhages in breast muscle. (*Peckham*). F. Hemorrhages in proventriculus. (*Peckham*).



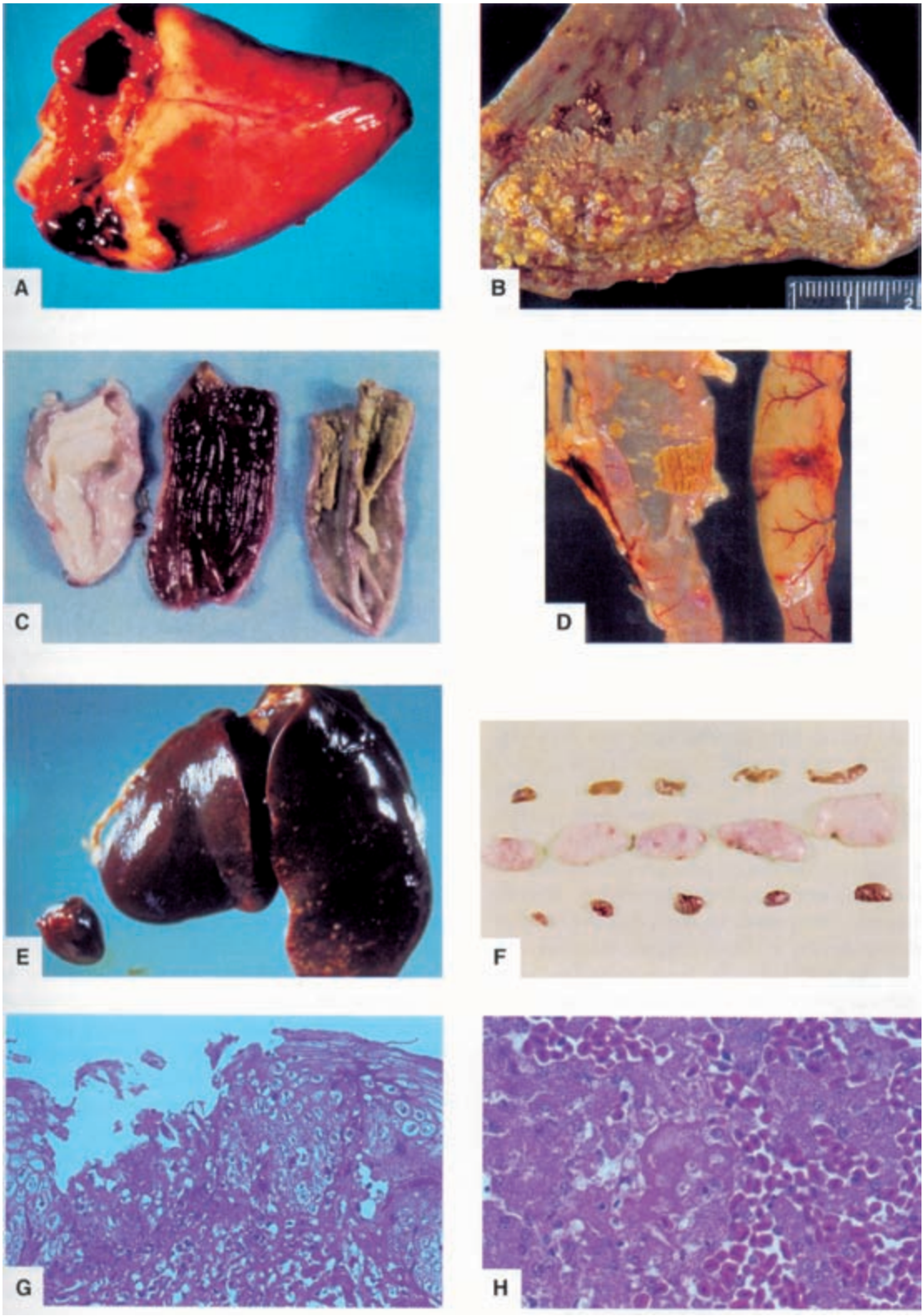
9.11 A–E. Quail bronchitis. A. Trachea from a young quail chick infected with quail bronchitis virus (QBV). There is opacity of the trachea due to the presence of necrotic exudate. B. Cross section of trachea from a young quail chick infected with QBV. The mucosa of the section on the left is extremely thickened, causing partial obstruction, and the section on the right is minimally affected. C. Microscopic section of trachea from a QBV-infected quail. There is epithelial deciliation, cell swelling, necrosis, desquamation, and leukocyte infiltration. D. Quail chick infected with QBV. The lungs are congested and contain red consolidated areas surrounding the bronchial hilus. E. Microscopic section of pulmonary bronchus from a quail infected with QBV. There is epithelial cell proliferation, leukocyte infiltration, and luminal exudate. Basophilic intranuclear inclusions are within epithelial cells. F, G. Hemorrhagic enteritis. F. Turkey, 7 weeks old. Duodenal loop is dark purple because of bloody contents (one section opened to show contents). Note splenic enlargement and mottling. There is also inflammation of a thoracic air sac (left) typical of acute colisepticemia, which often follows hemorrhagic enteritis virus (HEV) infection. G. Markedly enlarged and mottled spleen in turkey with HEV infection. (Barnes)



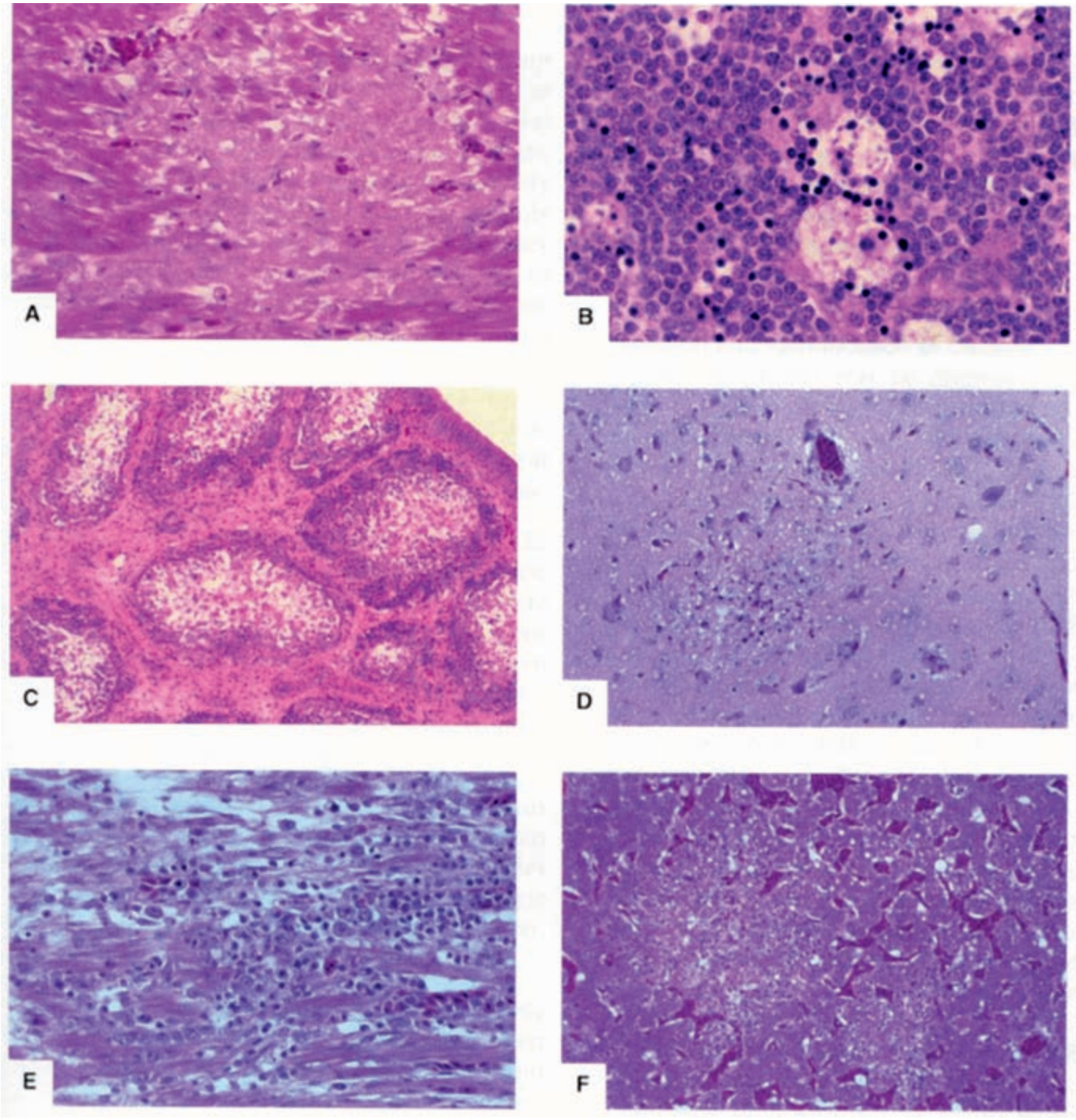
10.2. A. Cutaneous FPV lesions on the comb of a chicken. (Shivprasad). B. Diphtheritic FPV lesions in the mouth of a chicken (Shivprasad). C. Cutaneous fowlpox virus lesion on the eye and nostril of an experimentally infected chicken. D. Pocks (arrows) produced by FPV on the chorioallantoic membrane of developing chicken embryo. E. Microscopic examination of a cutaneous lesion produced by canarypox virus. Eosinophilic cytoplasmic inclusion bodies (arrows) are present in most of the infected cells. Infected cells are enlarged, and some infected cells have lost their nuclei. F. Microscopic examination of a section of "pock" (as seen in D) stained with Acridine Orange (AO). Cytoplasmic inclusion bodies containing DNA stain green (arrows) with AO.



13.1. A. Normal 15-day-old chick embryo (right). Fifteen-day-old chicken embryo inoculated 6 days previously with duck hepatitis virus (DHV) type 1 (left). Note small size, hemorrhage, and edema. B. Duckling dead from infection with DHV type 1. Note typical opisthotonos. C. Liver with hemorrhagic lesions caused by DHV type 1 infection. D. Microscopic lesions in liver of duckling 24 hours after infection. Note massive liver cell necrosis and hemorrhage. H & E, ×1000. E. Microscopic lesions in liver of duckling 7 days after infection. Note extensive bile duct proliferation. H & E, ×250.



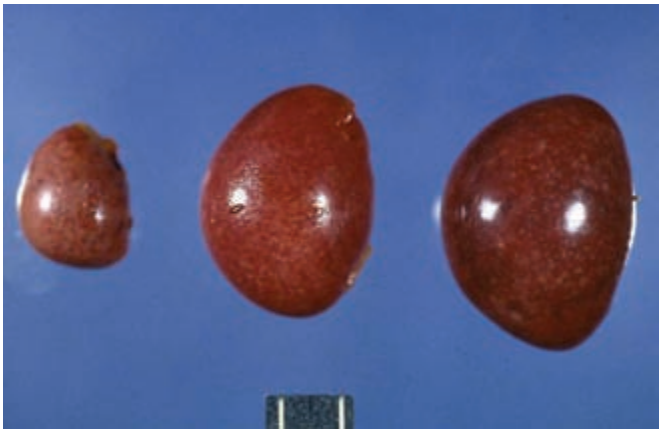
13.4. Duck virus enteritis (DVE) lesions in a duck. A. Petechial hemorrhages in the epicardium. (Munger). B. Extensive ulceration of esophageal mucosa. (Munger). C. Bursal lesions; hemorrhages (center) and cheesy exudate (right and left) (Shawky, Sandhu, Shivaprasad). D. Multifocal necrosis of gut-associated lymphoid tissue resulting in ulceration covered by fibrinous pseudomembranes. Note also the reddened ring visible on the external surface of the intestine. (Munger). E. Multiple pale focci in liver and slightly smaller dark-colored spleen. F. Normal thymus (middle) and thymus (DVE) showing hemorrhages and atrophy (top and bottom), 4 dpi (Shawky, Sandhu, Shivaprasad). G. Microscopic appearance of esophageal ulcerations. Not lack of inflammatory response and presence of intranuclear inclusion bodies. ×225. (Munger and Barnes). H. Microscopically, liver shows focal areas of necrosis filled with fibrin. Intranuclear inclusion bodies can be seen in hepatacytes near areas of necrosis. ×360. (Munger, Barnes).



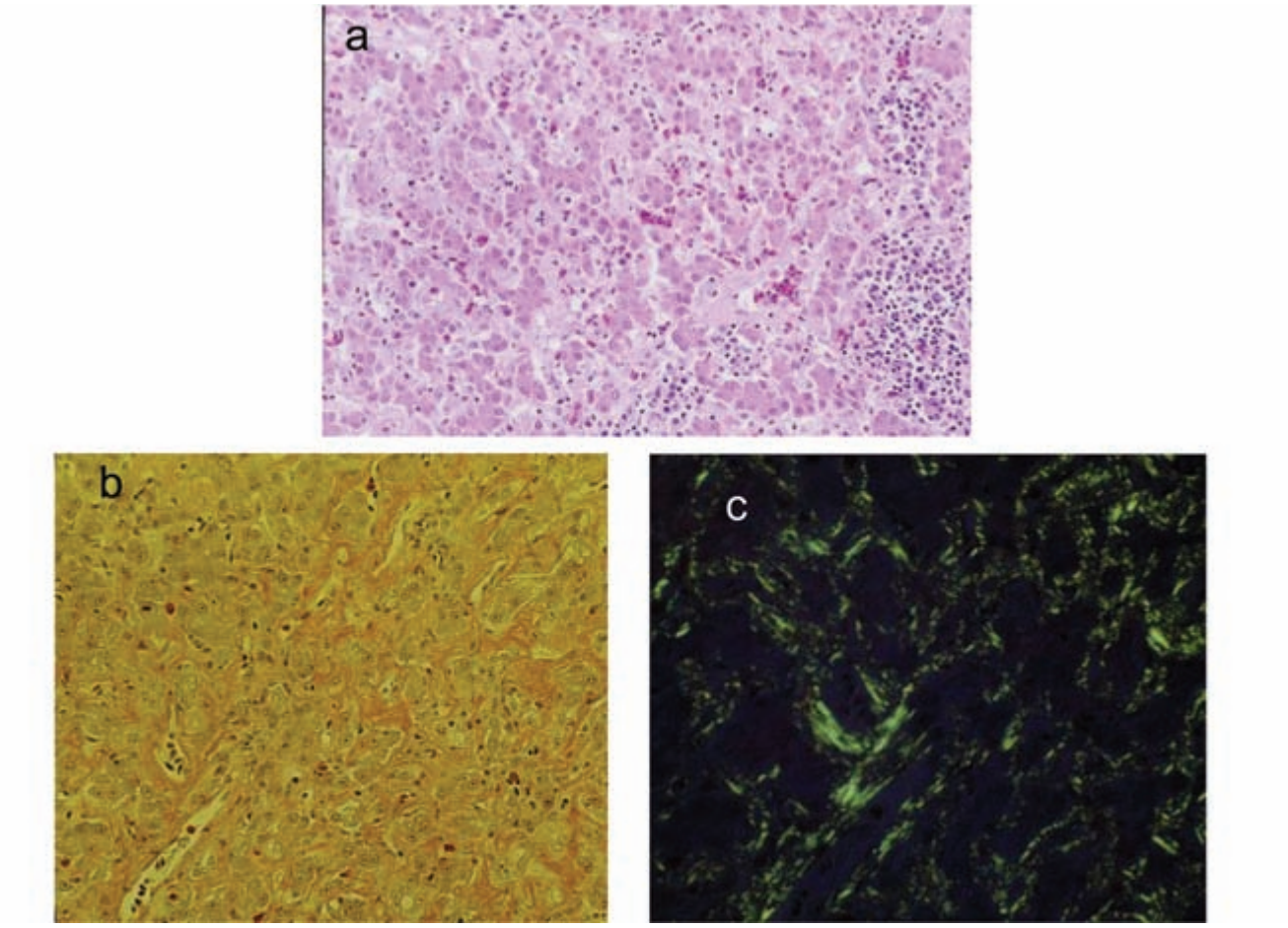
14.6. Microscopic lesions in turkeys and chickens experimentally infected with eastern equine encephalitis virus. A. Heart of turkey, 3 days postexposure. A large focal area of myocardial necrosis is present, with no inflammatory reaction. B. Thymus of turkey, 3 days postexposure. Aggregates of pyknotic nuclei within clear spaces indicate acute lymphocyte necrosis. C. Bursa of Fabricius of turkey, 3 days postexposure. Atrophy of bursal follicles with marked lymphoid depletion is present. D. Brain of chicken, 2 days postexposure. A focal area of necrosis is present with mild perivascular cuffing. Note emigration of mononuclear cells from an adjacent venule distended with erythrocytes. E. Heart of chicken, 5 days postexposure. Myocardial degeneration and necrosis with a mononuclear cell infiltrate. F. Liver of chicken, 5 days post-exposure. Focal necrosis is present with minimal inflammatory cell response.



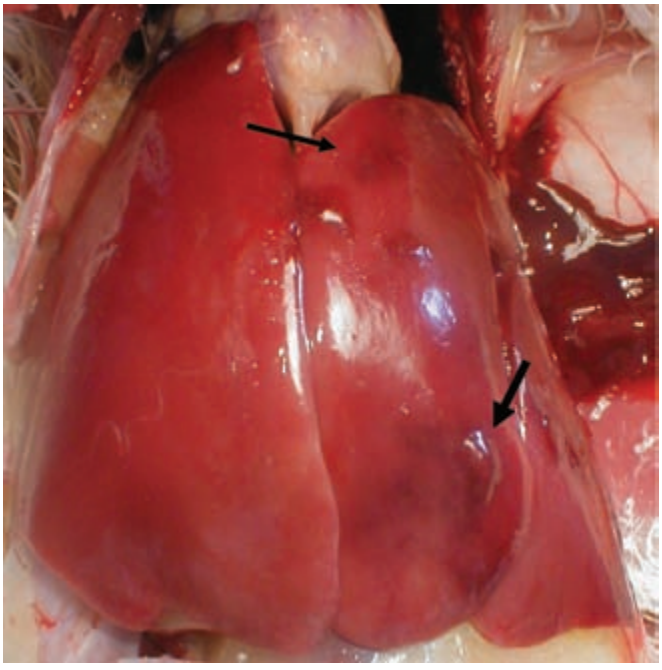
14.23. Enlarged and hemorrhagic liver from a 63-week-old chicken with hepatitis-splenomegaly syndrome. Note the liver is not fatty.



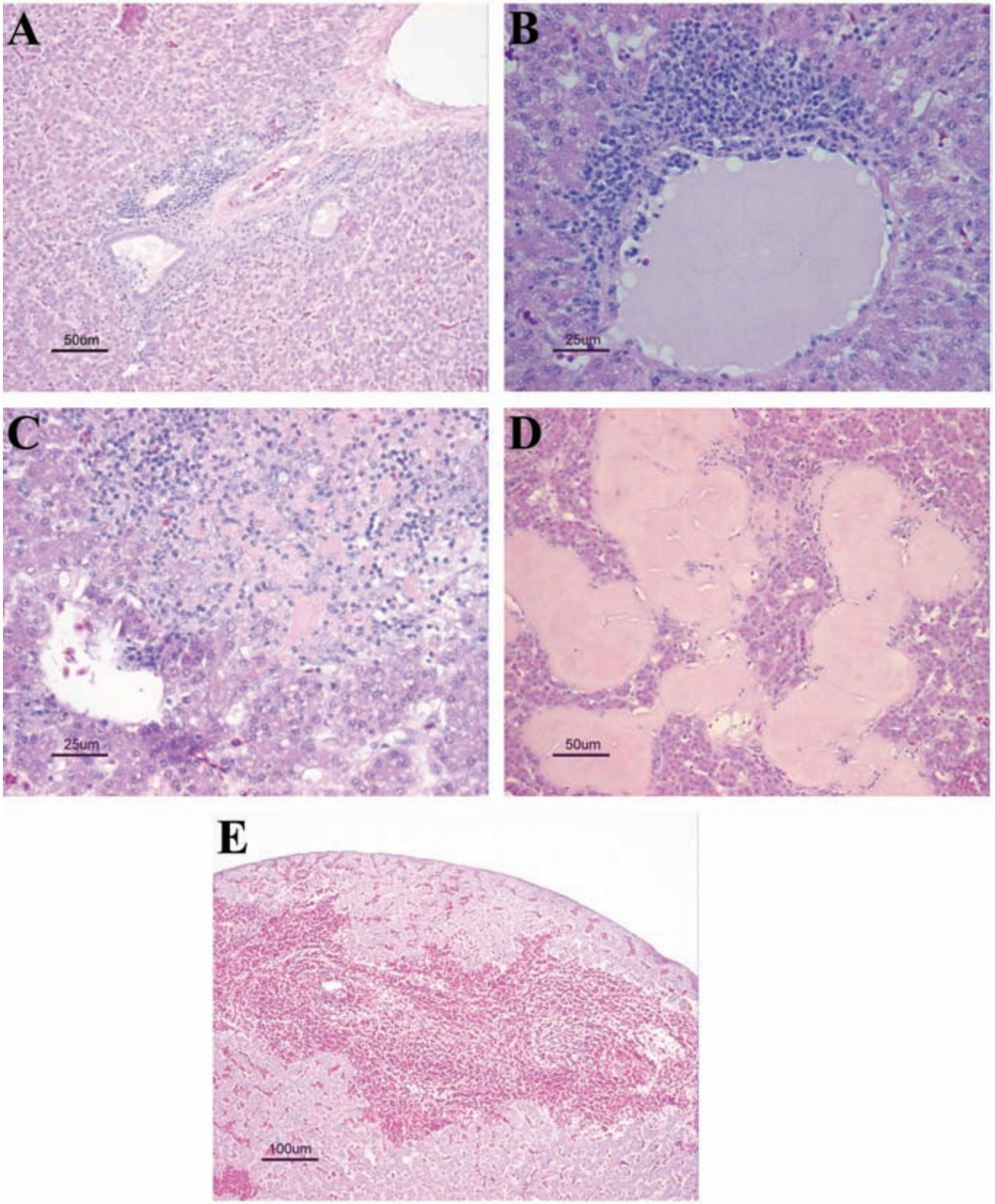
14.24. Two enlarged and mottled white spleens from 56-week-old chickens with hepatitis-splenomegaly syndrome. The spleen on the left is of normal size.



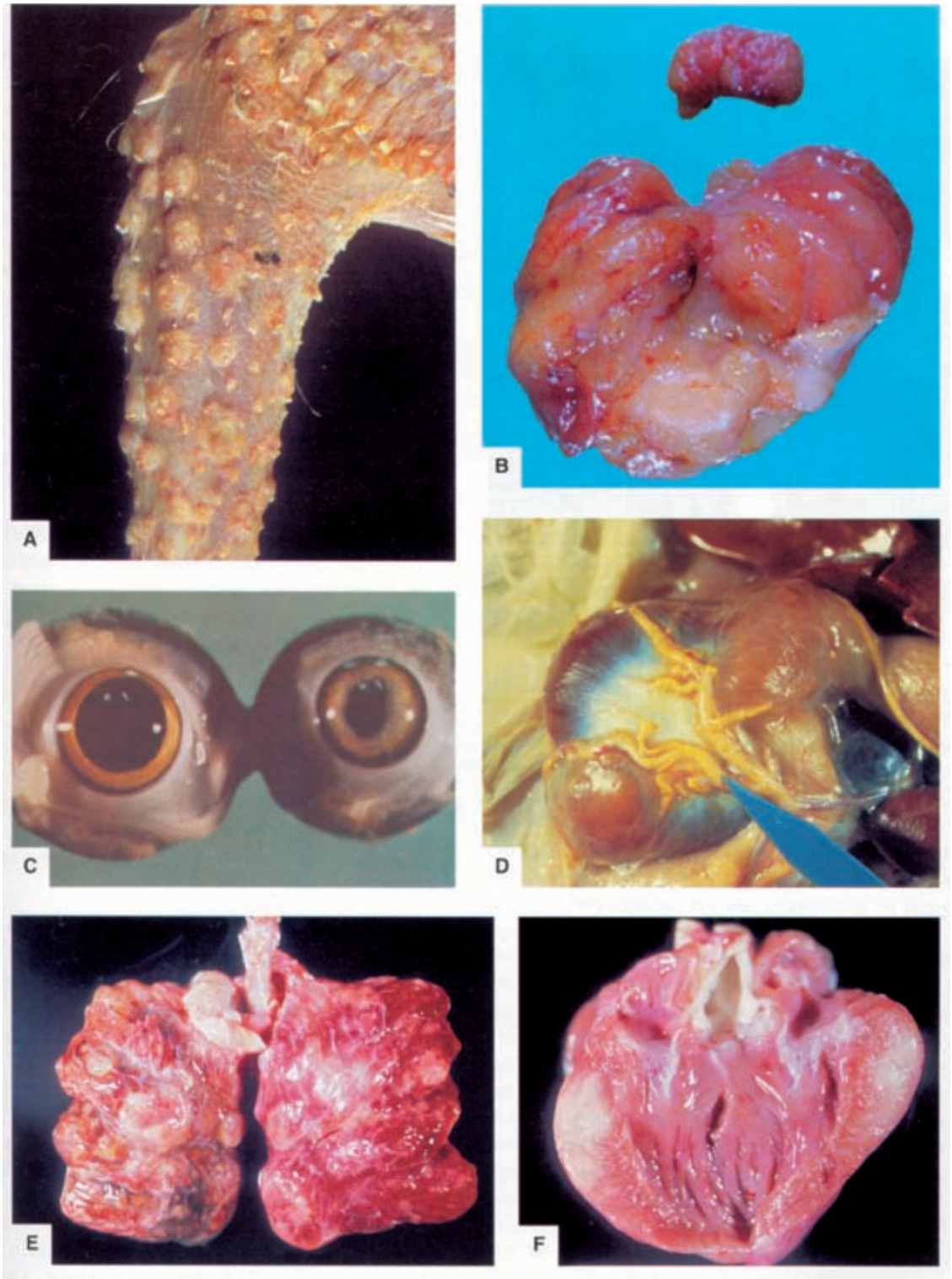
14.25. Photomicrographs of a liver from a chicken with spontaneous case of hepatitis-splenomegaly syndrome showing accumulation of homogeneous eosinophilic material, amyloid in the interstitium stained with H and E (a), Congo red stain positive shows orange colored amyloid (b), and apple green birefringence property of amyloid under polarizing filter (c).



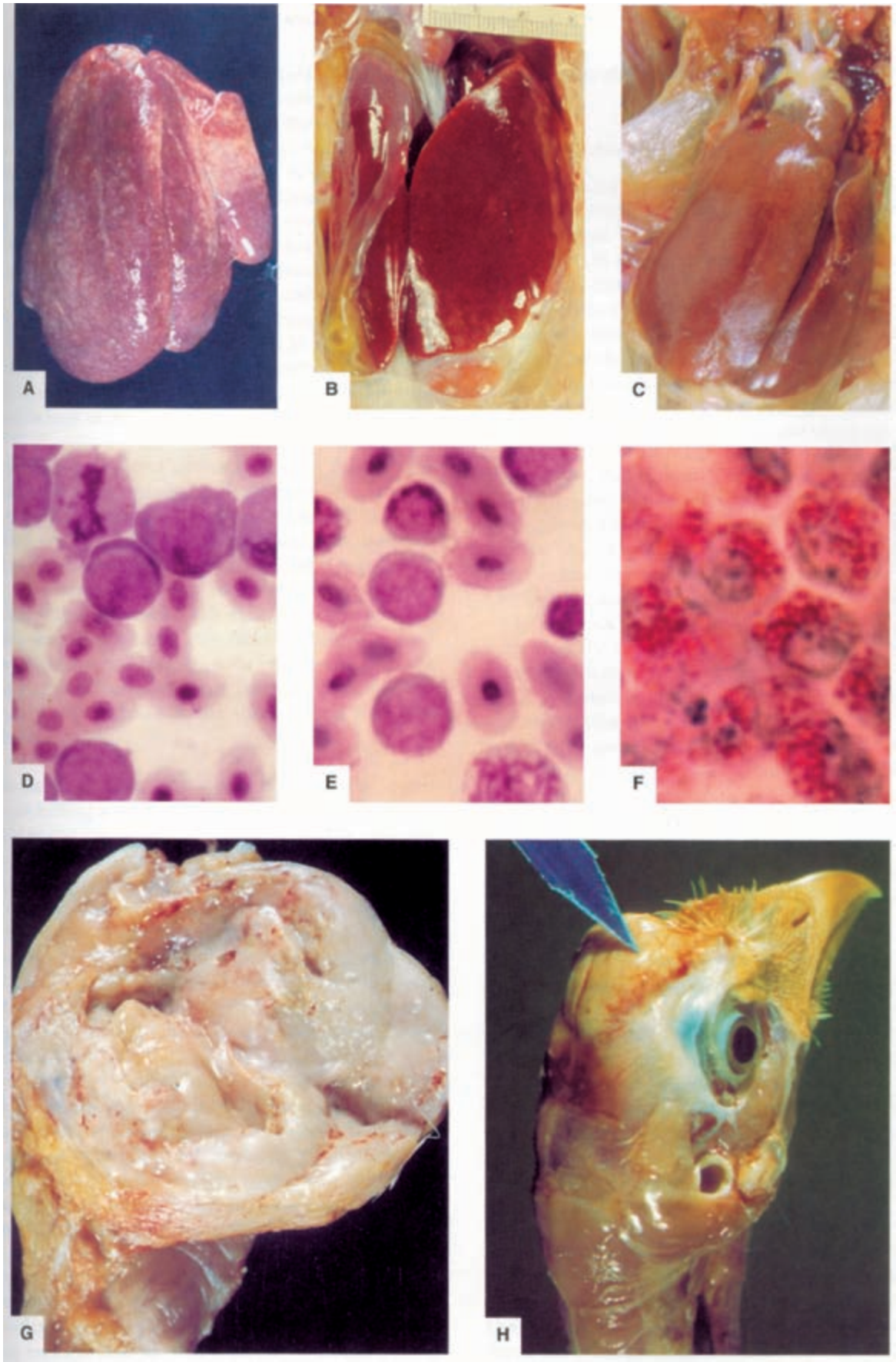
14.26. Gross lesion of a liver from a specific-pathogen-free chicken experimentally infected with avian HEV showing sub-capsular hemorrhages (arrows). Reproduced with permission from American Society for Microbiology (4).



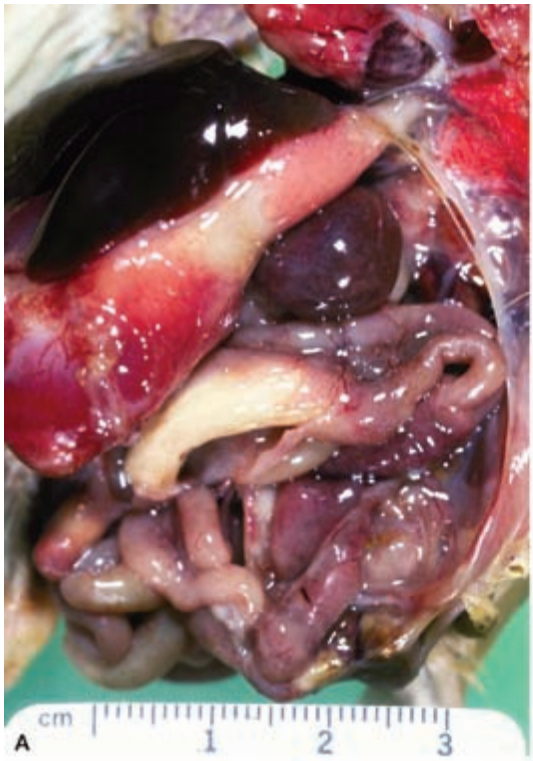
14.27. Microscopic lesions of the liver from chickens experimentally infected with avian HEV. A. A liver section from an oronasally-inoculated chicken, showing lymphocytic and scattered heterophilic portal vein periphlebitis. B. A liver section from an IV-inoculated chicken showing focally intense lymphocytic venous phlebitis and periphlebitis. C. A liver section from an IV-inoculated chicken showing locally extensive hepatocellular necrosis with lymphocytic inflammatory cell infiltration. D. A liver section from an IV-inoculated chicken. Note architectural disruption and coalescing deposition of hypocellular homogenous eosinophilic matrix with displacement of hepatocellular cords. E. A liver section from an oronasally-inoculated chicken. Note large focus of acute hemorrhage with local architectural disruption of hepatocellular cords and hepatic sinusoids. H&E staining. Reproduced with permission from American Society for Microbiology (4).



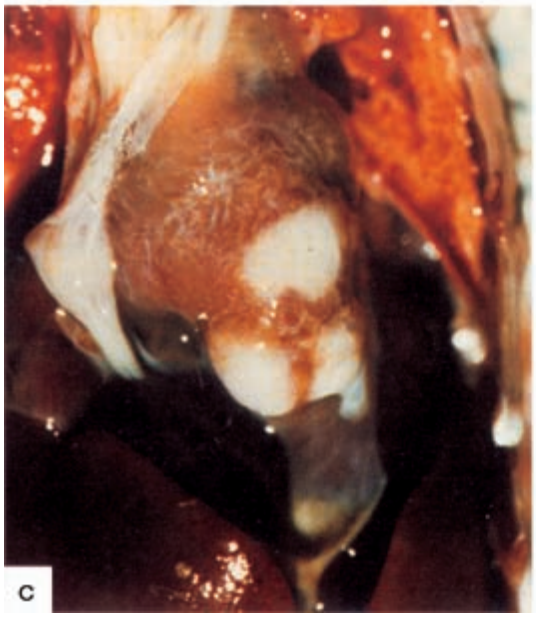
15.9. A. Leukotic tumors involving feather follicles (skin leukosis). (Peckham) B. Experimentally induced Marek's disease (MD) lymphoma in immature ovary (bottom) compared with normal ovary (top). (Whitter) C. Ocular lesions of MD. Note that the normal eye (left) has a sharply defined pupil and well-pigmented iris. Affected eye (right) has a discolored iris and very irregular pupil as a result of mononuclear cell infiltration. (Peckham). D. Gizzard from a chicken infected with CU-2 isolate of MDV. Note the grossly obvious atherosclerotic change in the arteries. (C. Fabricant). Microscopic changes from similar arteries are shown in Fig. 15.18B. E. Multiple lymphomas in lungs. F. Multiple lymphomas in heart. (Shivaprasad)

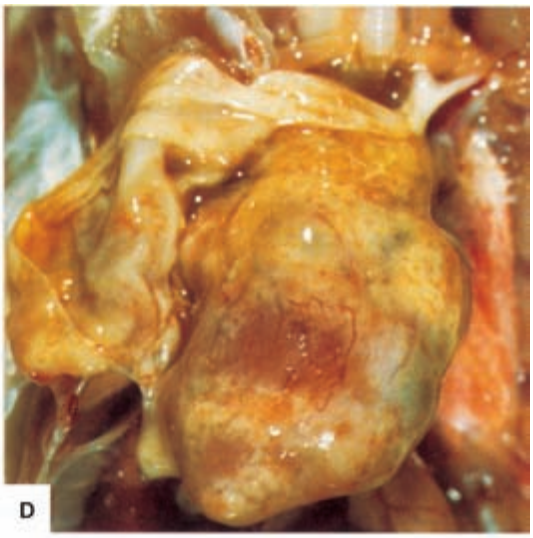


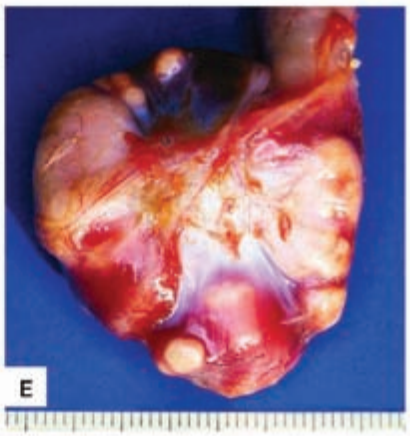
15.33. Comparison of leukosis. A. Lymphoid leukosis (LL). Diffuse form affecting the liver. Lesion is grossly indistinguishable from those in Marek's disease. B. Erythroblastosis. Enlarged cherry red liver and spleen. Note the fibrinous exudate. C. Myeloblastosis. Note enlarged gray-red liver. D. Erythroblastosis. Note basophilic cytoplasm and perinuclear halo. Blood smear, Giemsa, ×975. E. Myeloblastosis. Myeloblasts are slightly smaller than erythroblasts; cytoplasm is not as basophilic, nucleus is less vesicular, and nucleoli are not as frequent or conspicuous. Blood smear, Giemsa, ×975. F. Myelocytomatosis. Note myelocytes packed with acidophilic granules. Section of tumor, Giemsa, ×075. (Beard) G. LL tumors in the bursa of Fabricius (from the same bird as the liver shown in (A). H. Myeloid leukosis tumor on the surface of the skull. (Peckham)





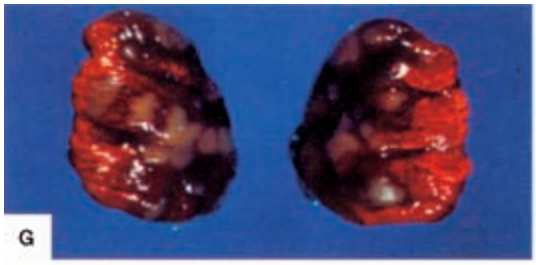








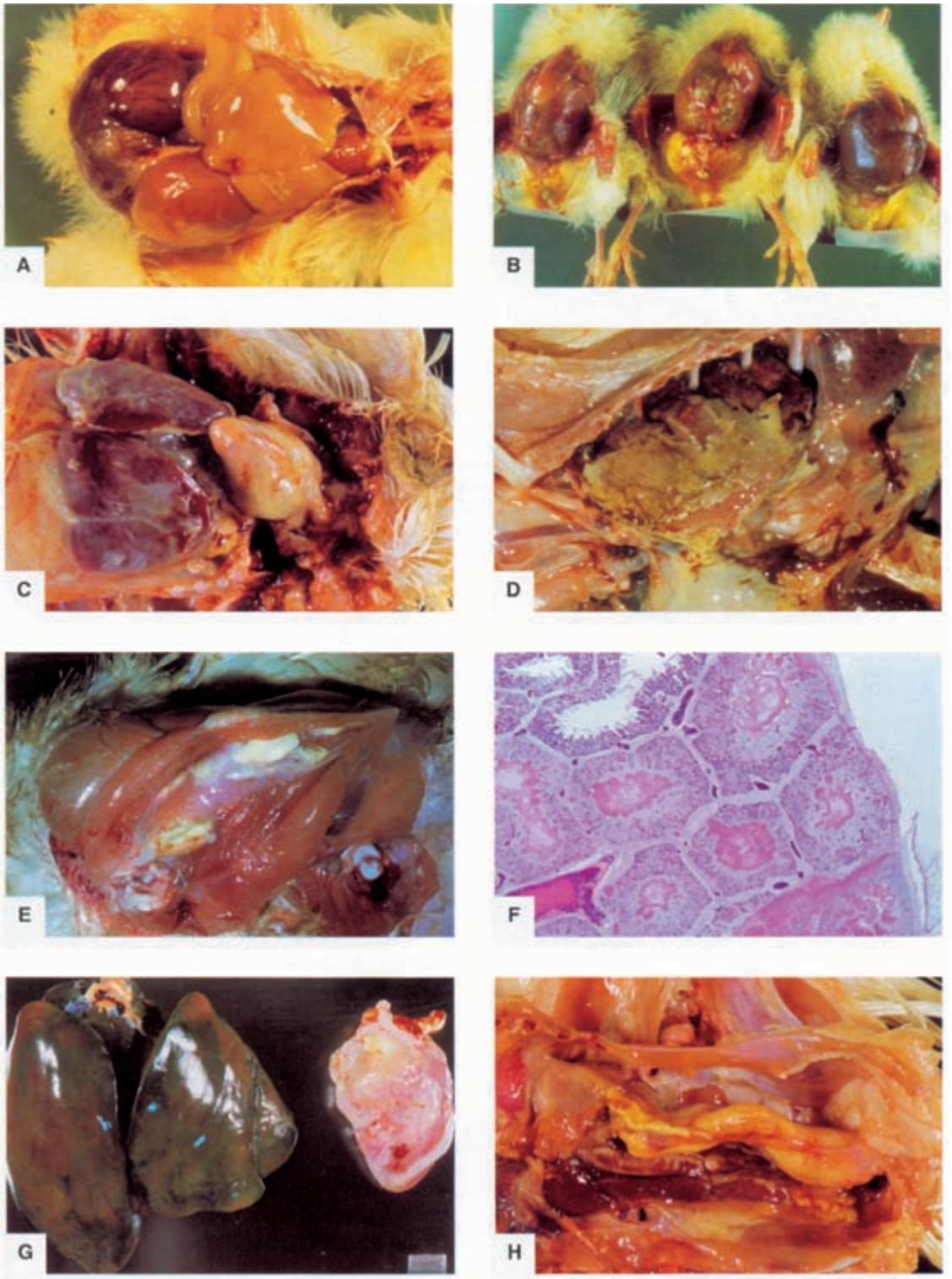
16.1. Gross lesions associated with Salmonella pullorum (A–I) infections in chicken. A. Enlarged and congested spleen and liver with pale yellow fibrinonecrotic cast in the lumen of cecum in a 10-day-old chick. B. Enlarged liver showing pale foci of necrosis (Glass). C. Heart from young chick with white nodules representing myocarditis. Such nodules can be confused for tumors, such as Marek's disease (Chin). D. Swollen hock joint containing yellow viscous fluid (Peckham). E. Ovarian lesions, salpingitis, and peritonitis. F. Lungs with pale exudate due to pullorum disease in a chick (Peckham). G. Nodular lesions in the heart due to chronic infection; note the thickened yellowish pericardium (reflected). H. Gizzard with yellow nodules of various sizes on the serosa in 6-week-old chick. I. Ovary with multiple misshapen grey nodular follicles.



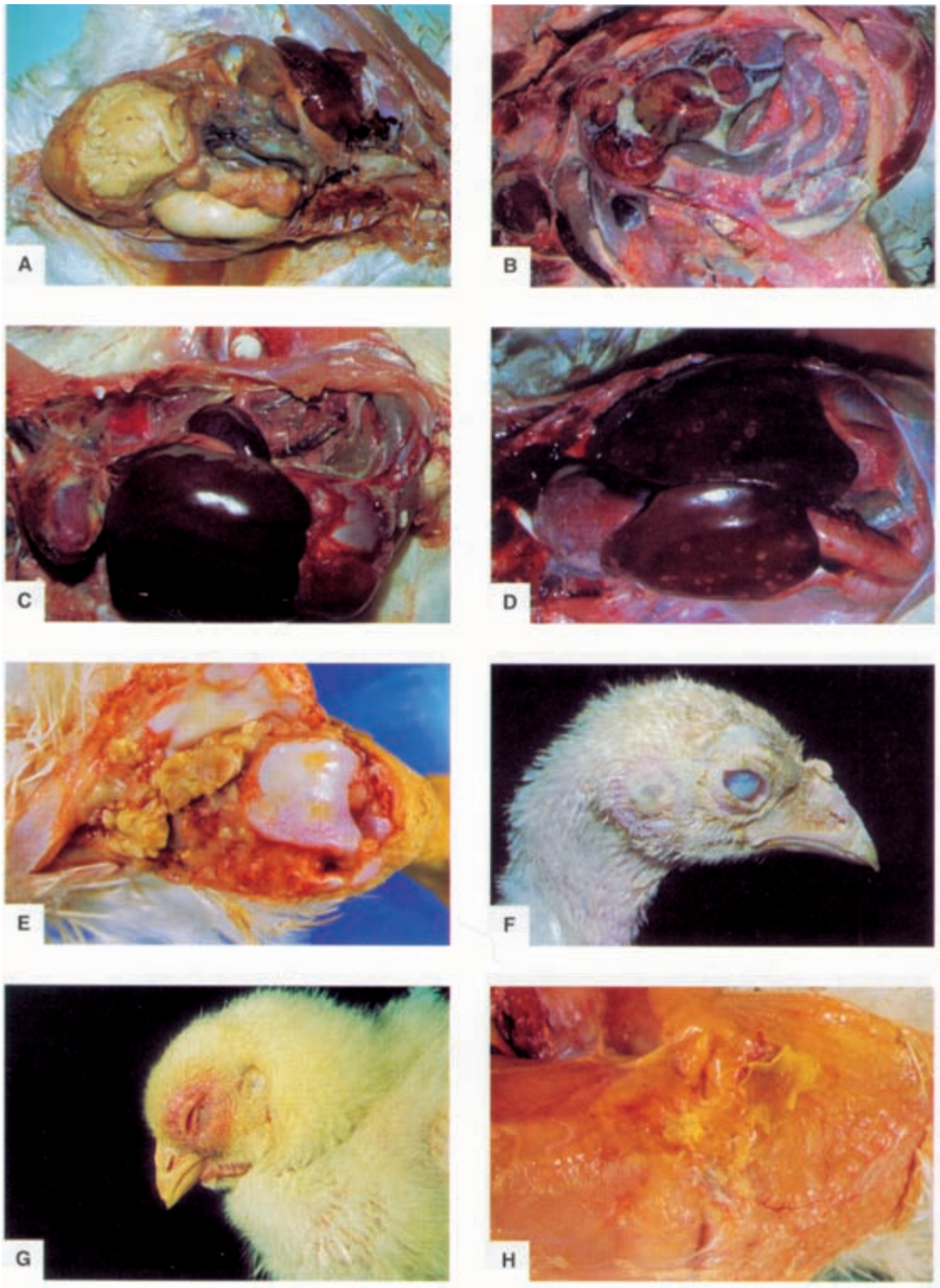




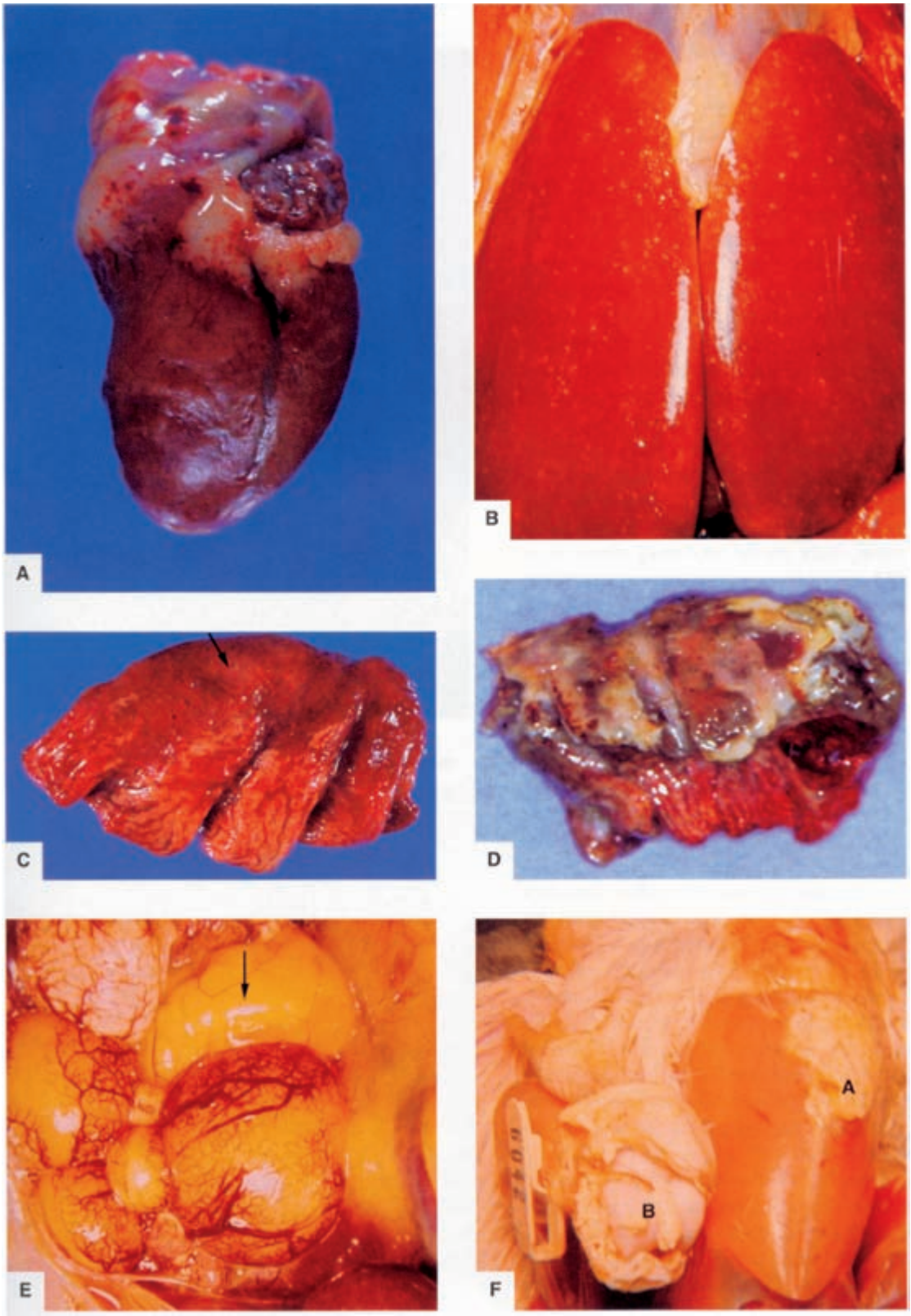
16.1 *(continued)*



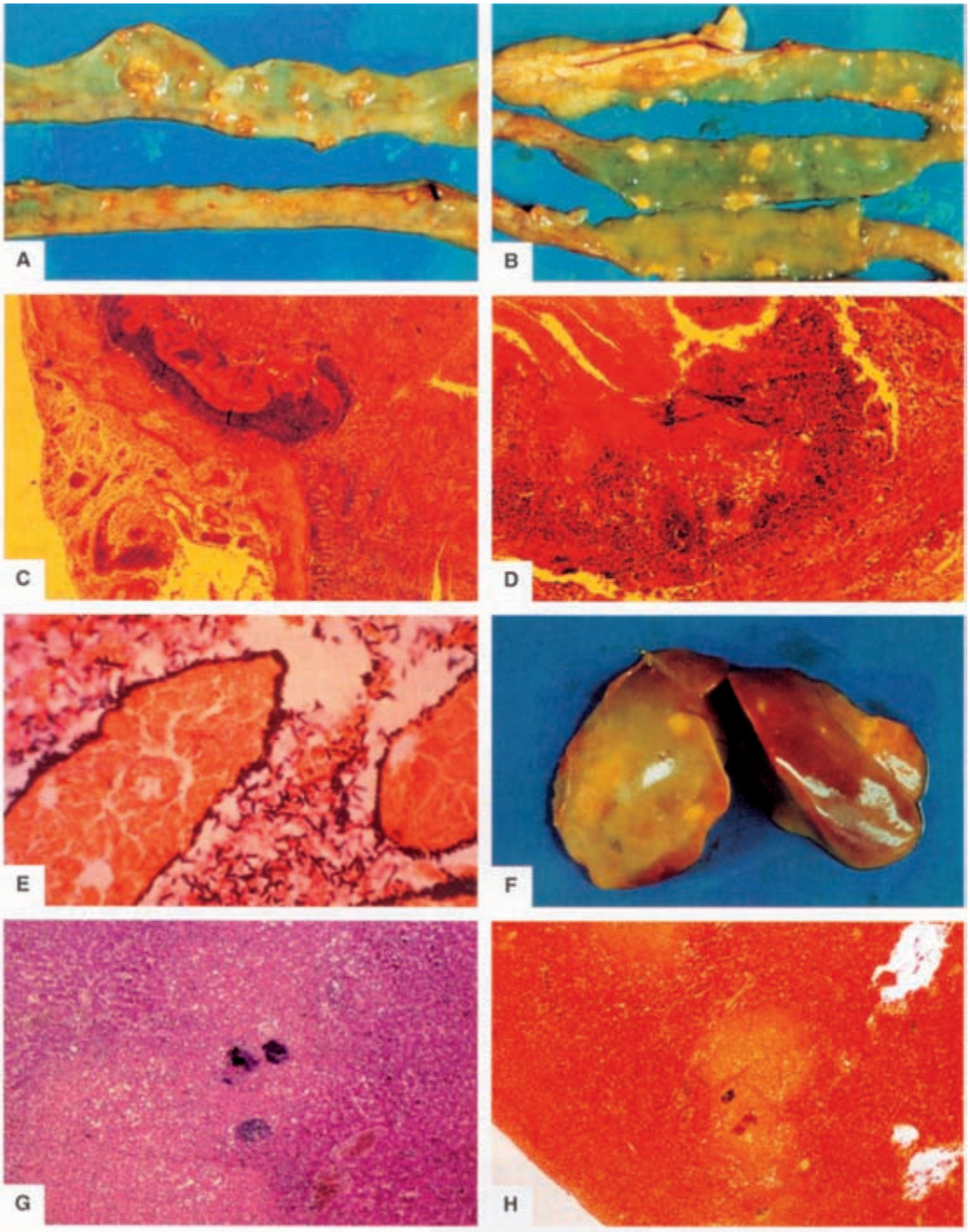
18.1. *Colibacillosis.* A. Yolk sac infection in a 4-day-old leghorn chick. Yolk sac is distended, hyperemic (note prominent vessels), and filled with abnormal brown, watery contents. B. Omphalitis and yolk sac infection in a group of 3-day-old leghorn chicks. Navels are inflamed, and yolk sacs are distended with abnormal contents. C. Advanced air sac disease in a 20-day-broiler chicken. Polyserositis (pericarditis, perihepatitis, peritonitis, airsacculitis) have occurred as a result of systemic spread of *Escherichia coli*. D. Pleuropneumonia and airsacculitis in a broiler chicken caused by *E. coli* infection. E. Experimental colibacillosis in a turkey. Extension of inflammation between superficial and deep pectoral muscles from airsacculitis involving the interclavicular air sac. Detecting this type of lesion is important during inspection at processing. F. Microscopic appearance of pneumonia caused by *E. coli* in a broiler chicken. Exudate fills the lumen of several affected parabronchi (compare with unaffected parabronchi at the top of the figure). Exudate has expanded some atria. Some atria have ruptured, permitting extension of the inflammatory process through the capillary bed into the interstitium. The process has involved almost all of one lobule with extension to the adjacent pleural surface. ×10. G. Pericarditis and green discoloration of the liver in a turkey that survived the acute septic phase of colibacillosis. Pericardium is thickened, and exudate in the pericardial sac is beginning to undergo fibrosis. Green discoloration of the liver can indicate inflammation elsewhere in the bird, especially in turkeys. H. Salpingitis in a young bird caused by *E. coli*. This lesion occurs infrequently but is often associated with airsacculitis involving the left abdominal air sac. (Figures A–C courtesy of Dr. L. Munger.)



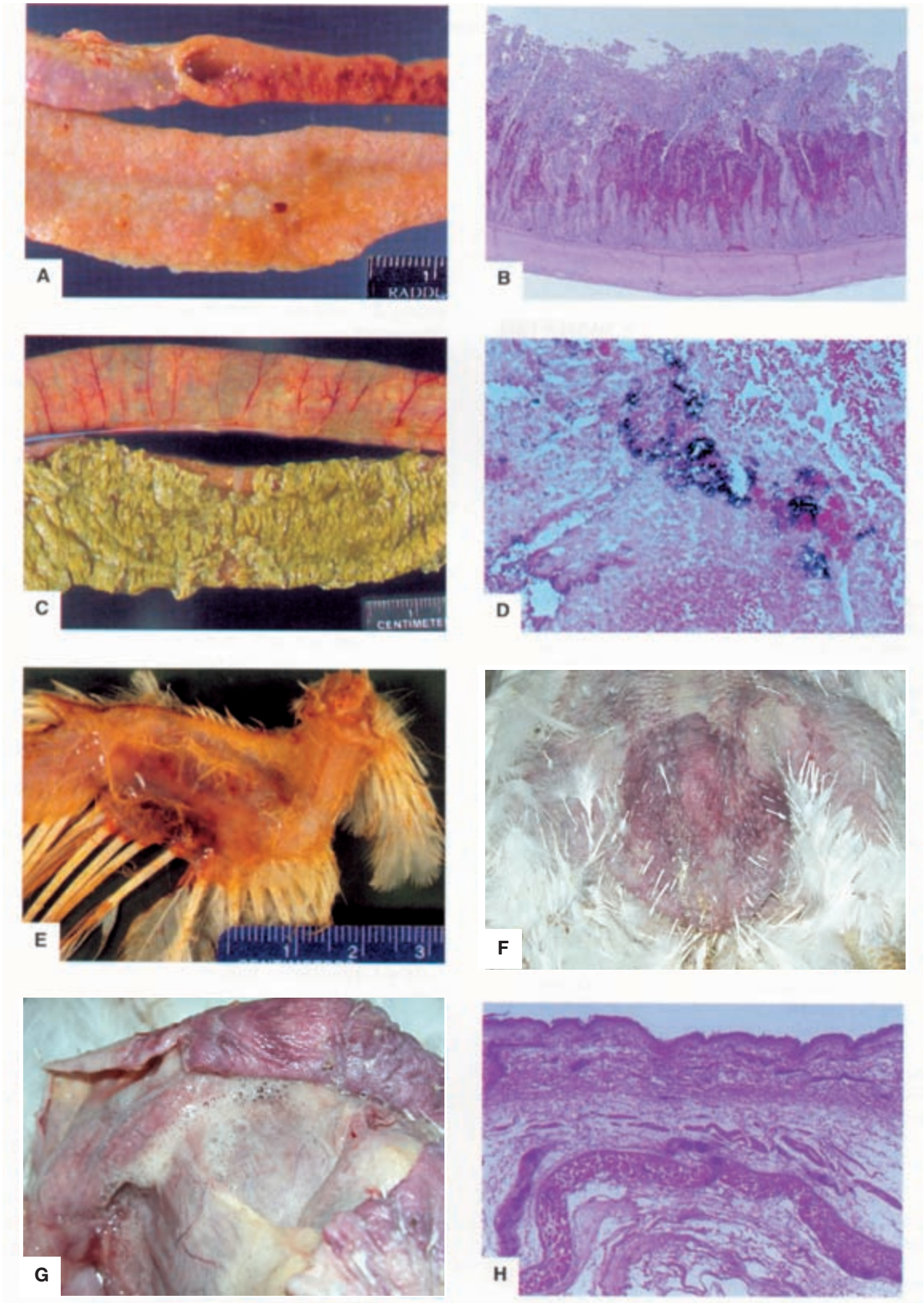
18.2. *Colibacillosis*. A. Large caseated masses distending the oviduct of this mature laying hen are characteristic of salpingitis caused by *Escherichia coli*. Salpingitis in the adult female most likely results from an ascending infection from the cloaca. B. Goose breeder with acute peritonitis. Yolk was demonstrated in the peritoneum and *E. coli* was isolated. C. Acute *E. coli* septicemia in a turkey. Spleen is markedly enlarged and congested. Note it is approximately the same size as the proventriculus. Liver is also enlarged and congested, and there is evidence of early pericarditis and peritonitis. D. Experimental colibacillosis in a turkey. Liver from a bird that survived the acute septicemic phase has multiple pale foci, which were determined microscopically to be focal areas of early heterophilic, granulomatous hepatitis. E. Advanced tenosynovitis/arthritis involving the hock joint and flexor tendons of a lame commercial broiler. *E. coli* and *Staphylococcus* spp. were isolated from the lesion. F. Panophthalmitis affecting the eye of a turkey that survived an earlier episode of colisepticemia. This lesion is uncommon and affects only one eye. The organism can be isolated from the eye for an extended period after it is no longer present in other tissues. G. Swollen-head syndrome in a broiler chicken. There is conjunctival inflammation and periorbital swelling due to cellulitis. Evidence of exposure to high ammonia levels and infection with infectious bronchitis virus and *E. coli* were found in this flock. H. Avian cellulitis (inflammatory process). Subcutaneous yellow, caseous exudate is present over the abdomen of this affected bird. (Figures E, G courtesy of Dr. L. Munger)



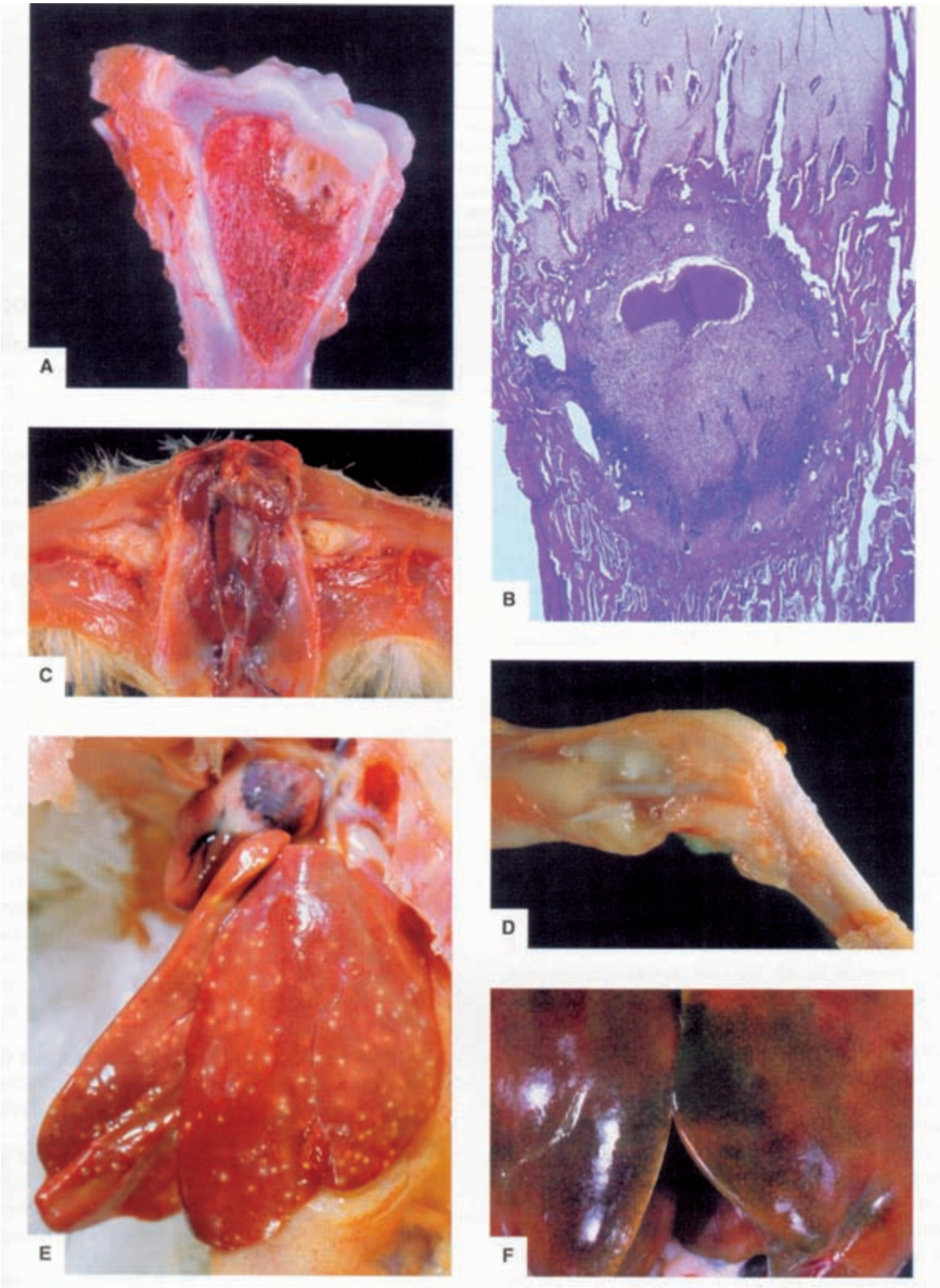
19.10. A. Acute FC; subepicardial hemorrhages in a turkey. B. Acute FC; multiple necrotic foci in turkey liver. C. Acute FC; turkey lung with extensive hemorrhage and patchy areas of necrosis (arrow) and emphysema. D. Submassive necrosis with fibrous exudate on pleural surface. E. Acute FC; flaccid ovarian follicle (arrow) with thecal blood vessels less evident than normal. F. Chronic FC; caseous exudate in sternal bursa (A) and hock joint (B) of a turkey.



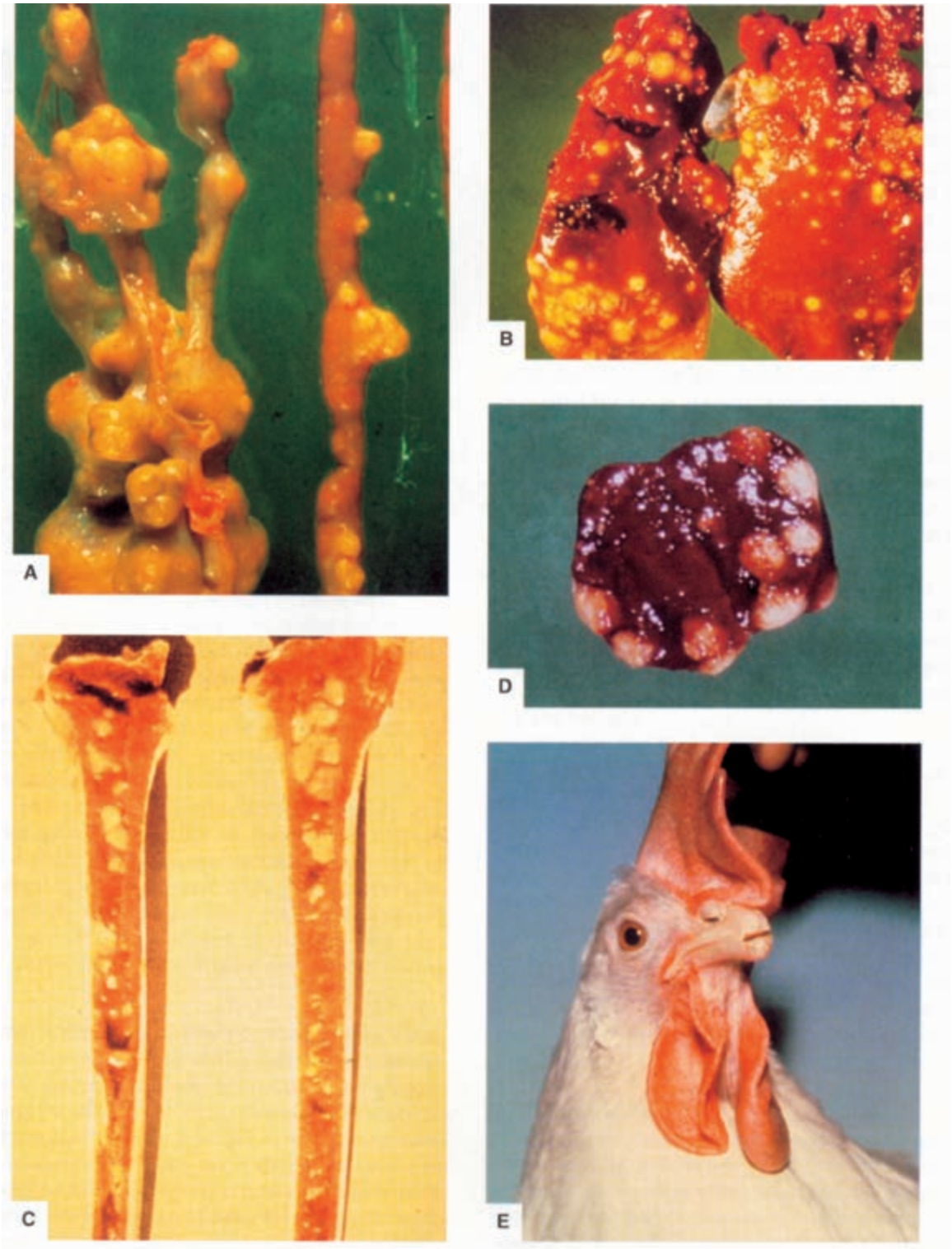
22.2. Ulcerative enteritis lesions in quail. A. Early intestinal ulceration visible from both mucosal and serosal surfaces. Note the hyperemia around some lesions and occasional ulcers with marginal hemorrhage. B. More advanced ulcers filled with diphtheritic, necrotic membranes. C. Transmural ulcer containing necrotic tissue and inflammatory exudate with adjacent focal peritonitis. D. Higher magnification of C. Note the sharp demarcation between viable and necrotic tissue by a zone of inflammation, inflammatory exudate, and bacterial colonies. E. Gram-stained, high magnification of inflammatory zone showing numerous large gram-positive bacilli typical of *Clostridium colinum.* F. Areas of necrosis in chicken liver. G. Microscopic appearance of liver lesion. Note the spreading areas of necrosis without distinct separation for normal tissue, minimal inflammatory response, and bacterial colonies. H. Gram stain of liver lesion. Note gram-positive staining colonies in necrotic foci.



22.4 A. Mild to moderate necrotic enteritis in a 7-week-old broiler breeder chicken with concurrent coccidiosis. Note the hyperemia and diffuse necrosis of the mucosa with multifocal ulcerations. (Dr. Laddie Munger) B. Intestine of a turkey showing uniform diffuse coagulation necrosis of mucosa. Deeper viable mucosal tissue is demarcated from necrotic luminal mucosal tissue by a zone of intense hyperemia, hemorrhage, and inflammation. ×20 (Dr. John Barnes) C. Severe necrotic enteritis in a commercial broiler. Note the "Turkish towel" appearance to the necrotic pseudomembrane covering the intestinal mucosa. (Dr. Chuck Hofacre). D. Necrotic enteritis in a 6-week-old ostrich caused by *C. difficile*. Note the severe diffuse coagulation necrosis with separation from the underlying viable tissue by an intense zone of inflammation with numerous large Gram-positive bacilli located at the interface of the necrotic and viable tissue. (Drs. Laddie Munger and John Barnes) E. Gangrenous dermatitis affecting the wing of a 12-day-old broiler. Spontaneous separation of epidermis revealing edematous, hyperemic, acutely inflamed dermis. (Laddie Munger) F. Broiler, 6-week-old, with gangrenous dermatitis. Extensive discolored patches of necrotic skin are present on the abdomen. (H. John Barnes) G. Same bird as in (F). Skin reflected to show discolored muscle and serosanguinous fluid expanding underlying dermis. (H. John Barnes) H. Skin from a turkey with gangrenous dermatitis. Dermis beneath a normal epidermis is markedly expanded by fluid and gas. Cutaneous muscle is undergoing rhadomyolysis. Cellular changes are minimal to absent, ×13. (H. John Barnes)

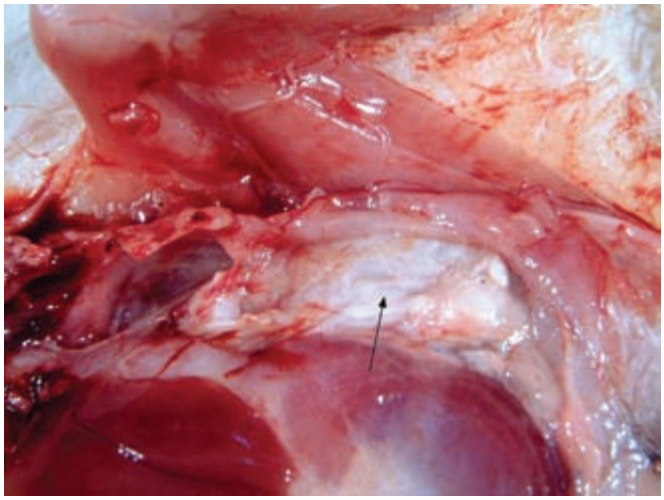


23.1. Lesions of staphylococcosis. A. Osteomyelitis of proximal tibiotarsus (×5). (H. John Barnes) B. Focal osteomyelitis subjacent of physis of proximal tibiotarsus (×5). (H. John Barnes) C. Bilateral osteomyelitis of femoral head due to *Staphylococcus aureus* infection in a 2-week-old turkey. Note the extension through the joint into the body cavity. D. Three-week-old turkey. Swollen hock joint with extension of inflammatory exudate along tendon sheaths. (Laddie Munger) E. Leghorn, 20 weeks old. Multiple foci of necrosis in liver following septicemic staph infection. (Laddie Munger) F. Green liver discoloration seen in turkeys wit osteomyelitis.
(H. John Barnes)

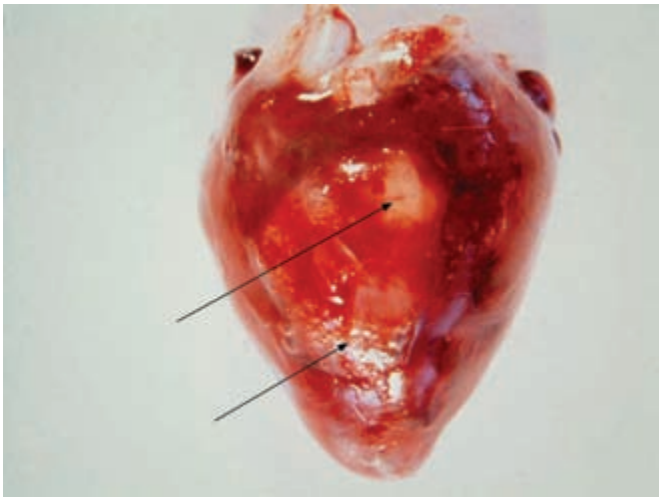


23.18. A–D. Tuberculous lesions in intestine (A), liver (B), bone marrow (C) (Peckham); and spleen (D) of naturally infected chickens. Note the variation in size of granulomas in the liver and spleen. E. Positive reaction in the left wattle of tuberculous chicken 48 hours after intradermal infection of avian tuberculin. (M. C. Peckham)

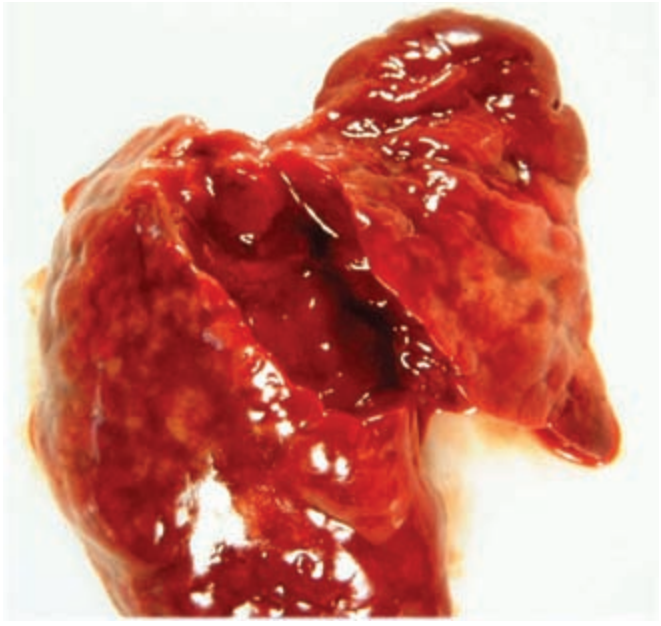
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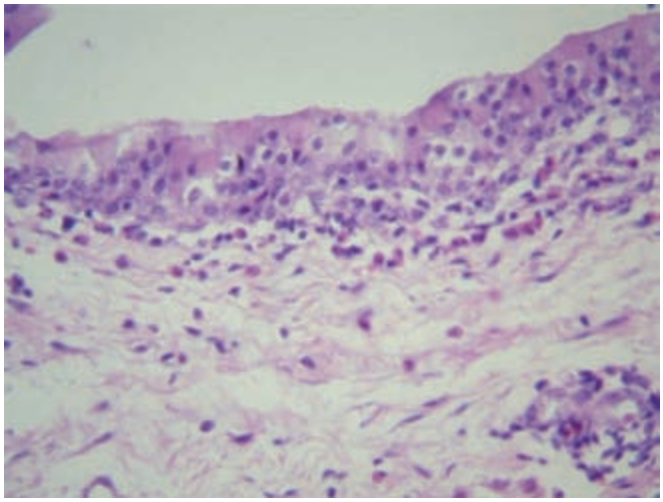


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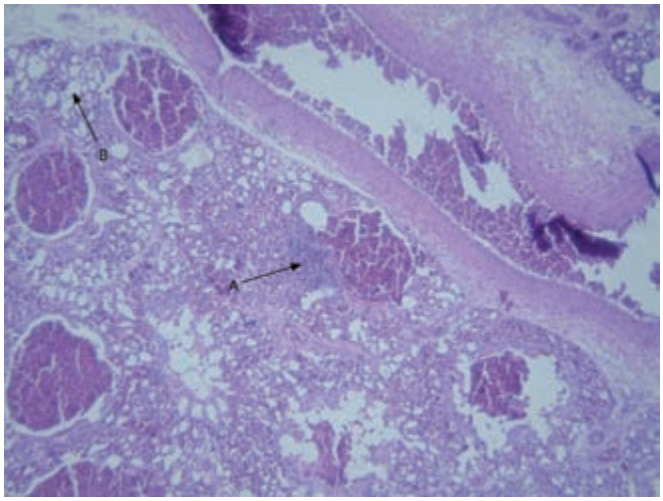


24.9. Turkey experimentally infected with a *C. psittaci* serovar A strain isolated from the lungs of a budgerigar. A. Notice the thickened abdominal air sac totally covered with fibrin plaques (arrow). B. Notice the presence of serous fluid and fibrin in the pericardial sac (arrows) together with C. severe hepatomegaly (C.).

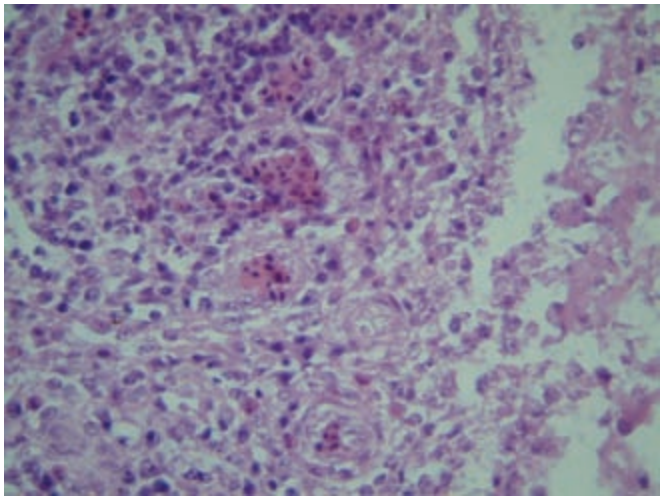
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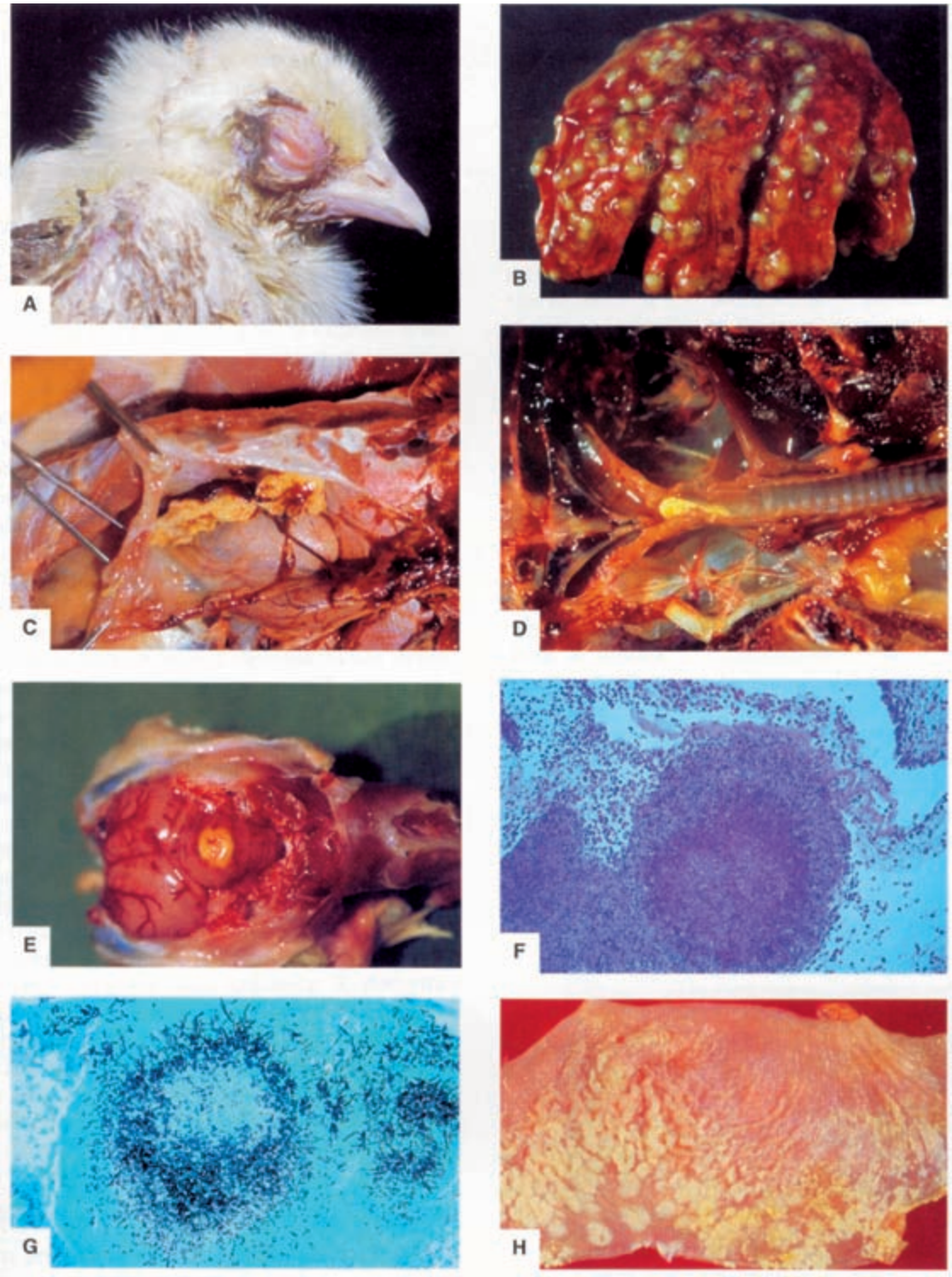
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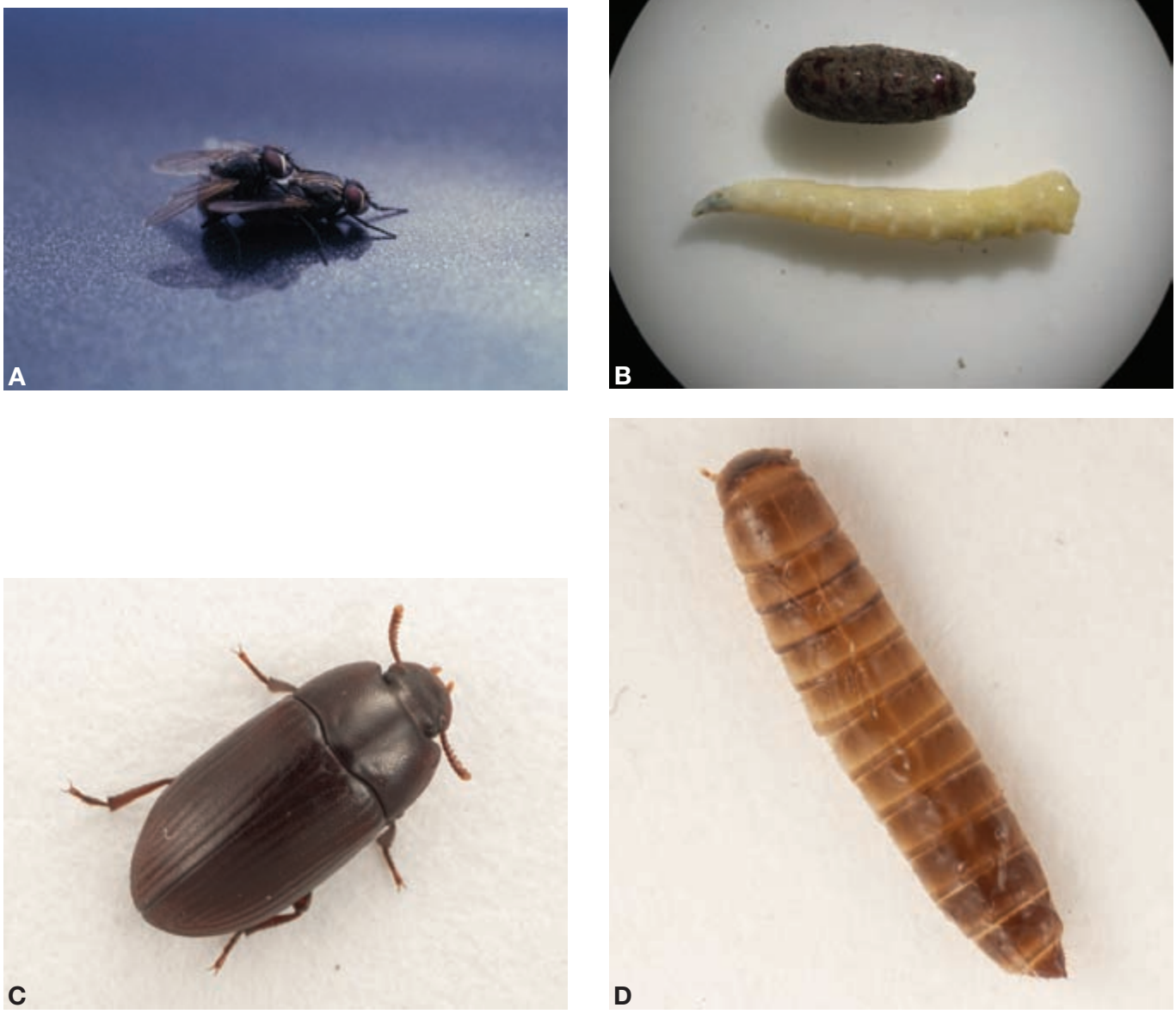
C



24.10. Hematoxylin and eosin staining of experimentally infected turkeys. A. Conjunctiva with infiltration of lymphocytes and heterophils together with epithelial vacuolization and hyperplasia (×172). B. Congested lung with infiltration of lymphocytes (arrow A) and dilated bronchi and parabronchi (arrow B) (×69). C. Fibrinous necrotizing airsacculitis (×172). .



25.3. Aspergillosis (A–G). Thrush (H). A. Ocular aspergillosis. This form is characterized by extensive keratoconjunctivitis. Panophthalmitis is another form of ocular aspergillosis in which internal structures, especially those in the posterior chamber of the eye, are affected. The latter is considered to result from hematogenous spread. B. Respiratory aspergillosis in the lung showing large and extensive caseous nodules. (M. C. Peckman) C. Caseous nodules due to aspergillosis in the air sac. D. Caseous exudate in the syrinx of a bird affected with aspergillosis. (M. C. Peckman) E. Mycotic encephalitis. Focal lesions in the brain can be extensive. F. Experimentally induced granulomatous lesion in the air sac due to aspergillosis. A central caseous core is bordered by a narrow, uniform palisade of macrophages and small giant cells surrounded by a less distinct broad zone of macrophages and scattered heterophils. ×90. (H. John Barnes) G. Gomori's methenamine silver-stained section of (F) demonstrating extensive black-staining fungus. ×90. (H. John Barnes) H. Candidiasis (crop mycosis). Crop is markedly thickened by a soft, yellow-white to gray irregular pseudomembrane, which has a curdlike-appearance. (M. C. Peckham)



26.1. Common poultry pests. A. Mating house flies (*Musca domestica*). B. House fly (*Musca domestica)* puparium and larva. C. Darkling beetle (*Alphitobius diaperinus*). D. Lesser mealworm (*Alphitobius diaperinus*).

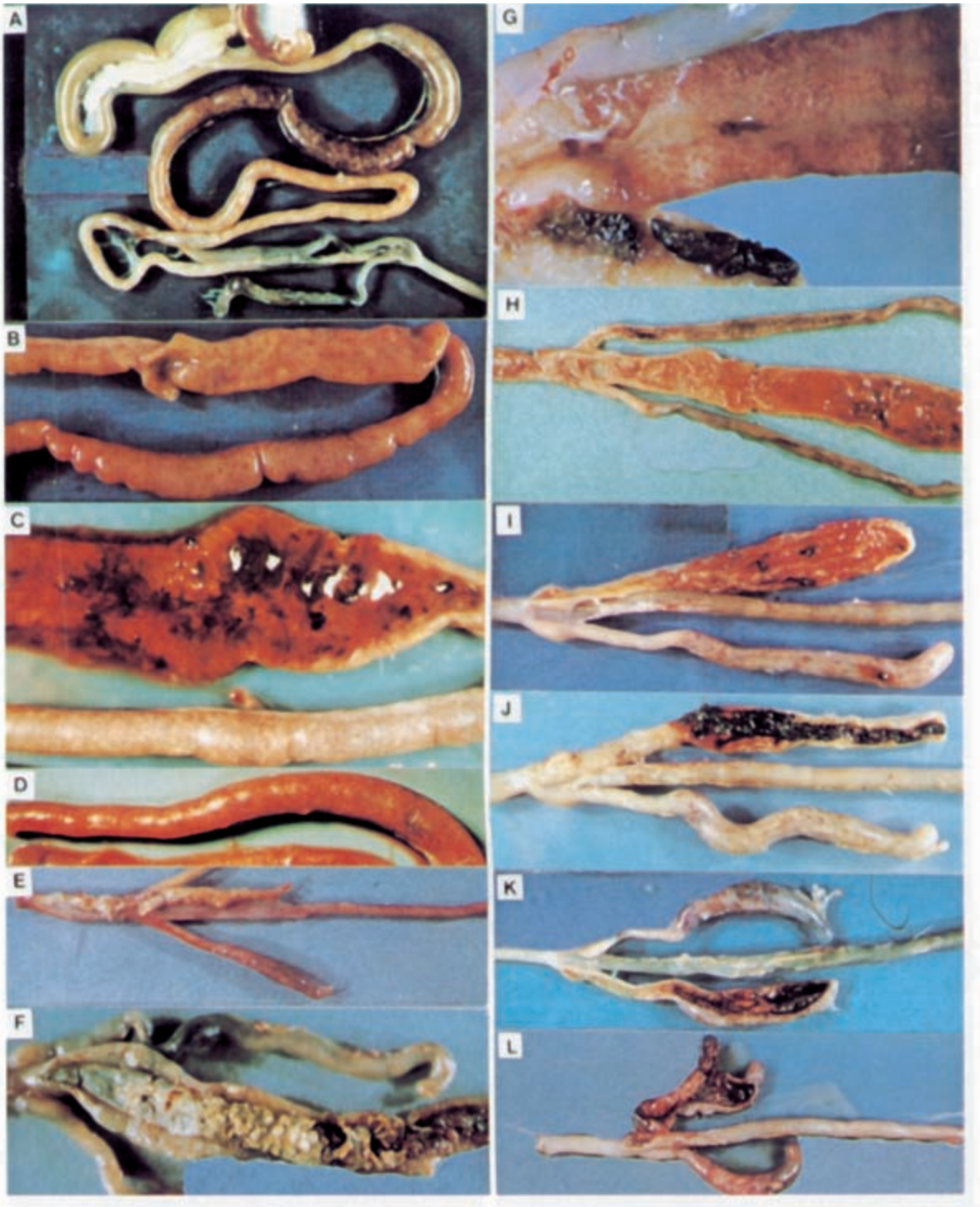




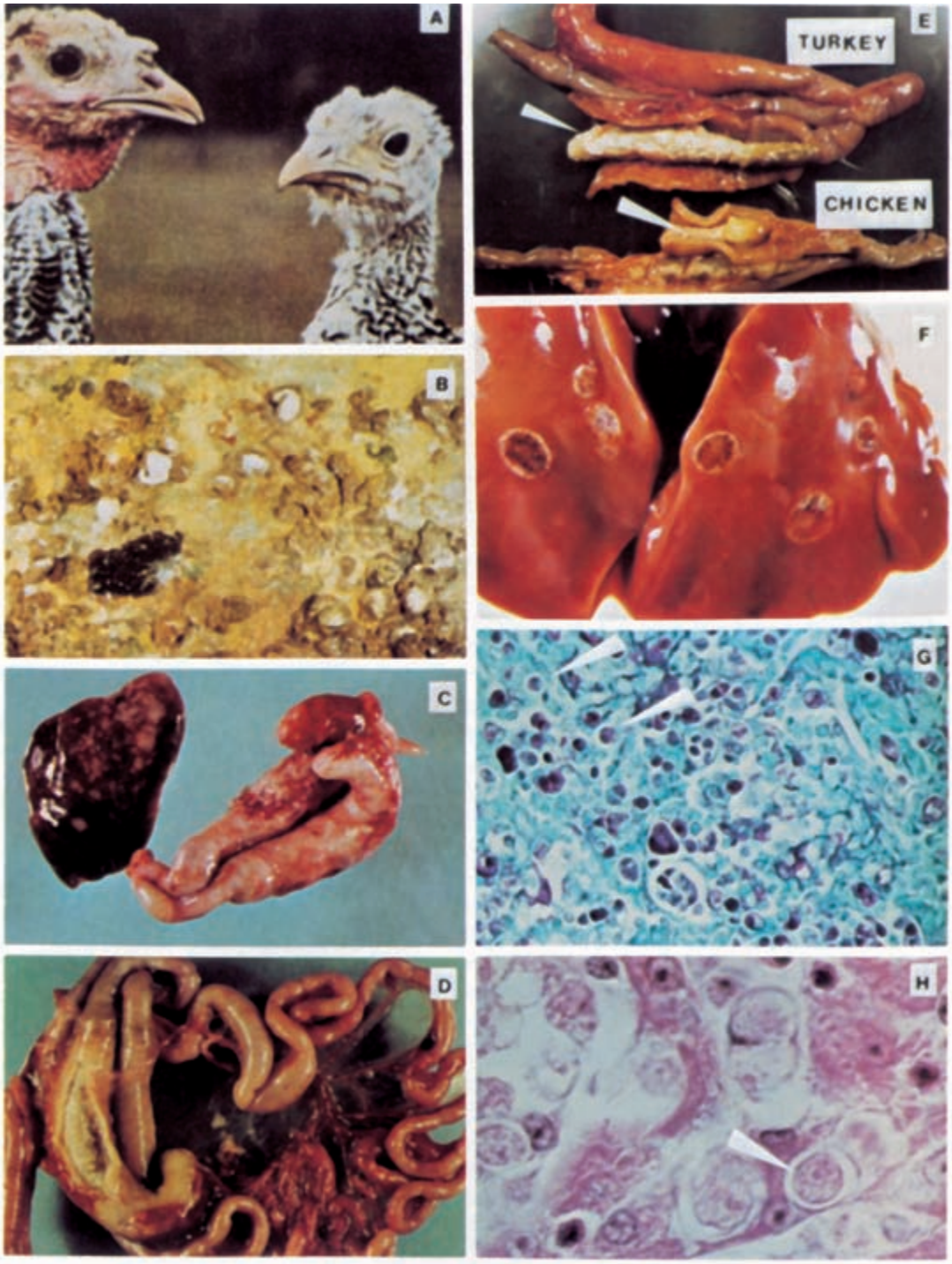
26.1. (*continued*) E. Chicken body louse (*Menacanthus stramineus*). F. Chicken body lice (*Menacanthus stramineus*). Photos A, B, E, F by Nancy Hinkle. Photos C, D by Aubree Roche.



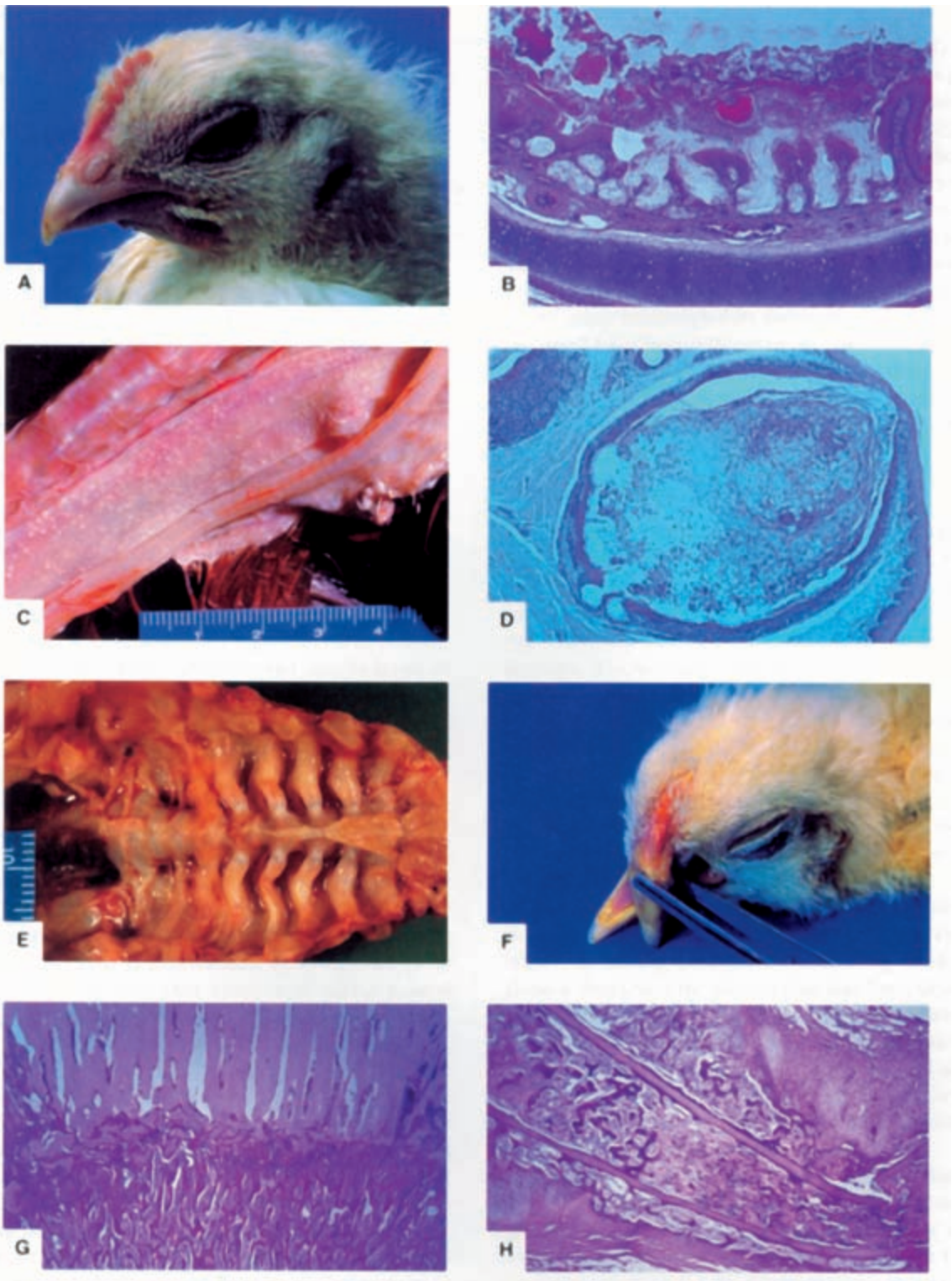
28.3. A. Oocysts and a microgametocyte (center) of *Eimeria maxima* (Long et al. [British] Crown copyright 1976). B. *E. acervulina* (+2). C. *E. acervulina* (+2). D. *E. acervulina* (+3). E. *E. acervulina* (+4). F. 1. Sporulated *E. maxima* with distinctive brownish walls; 2. Unsporulated *E. maxima* showing roughened outer wall; 3. Probably E. *tenella*; 4. End view, probably *E. mitis*; 5. Side view, probably two *E. mitis*. G. 1. normal midgut; 2. *E. maxima* midgut (+1). H. *E. maxima* midgut (+2 or +3) (Long et al. [British] Crown copyright 1976). I. *E. maxima* (+3). J. *E. maxima* close-up view (+4).



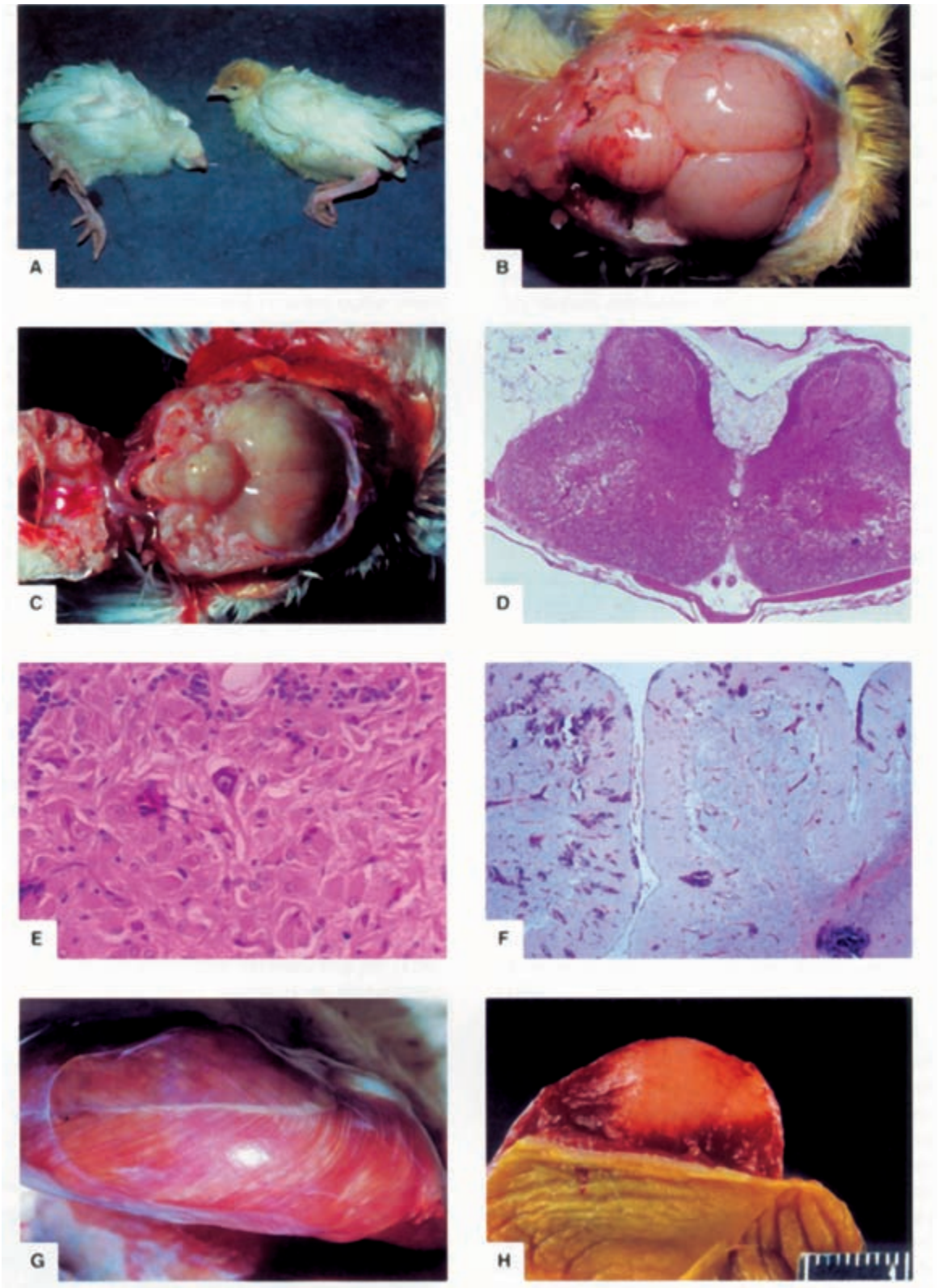
28.4. A. *Eimeria necatrix* showing ballooning in midgut. B. *E. necatrix* (+2). C. *E. necatrix* (Long et al. [British] Crown copyright 1976). D. *E. necatrix* (4+). E. *E. brunetti* (+4). F. *E. brunetti* (+4). G. *E. brunetti* (+3). H. *E. brunetti* (+4) (Long et al. [British] Crown copyright 1976). I. *E. tenella* (+2). J. *E. tenella* (+3). K. *E. tenella* (+4). L. *E. tenella* (+4) with cecal core.



28.11. A. Normal uninfected poult (left); histomoniasis-infected poult of the same age (right). Sickly appearance occurs later in the course of infection and is not distinctive to histomoniasis. (Hilbrich) B. Brilliant yellow material in feces often constitutes the first sign of histomoniasis outbreaks in turkeys. C. Liver and cecum from poult 14 days after feeding on *Heterakis gallinarum* ova. Note engorgement of ceca and diffuse nature of liver lesions. (McDougald) D. Intestinal tract from experimentally infected turkey showing engorged cecum, core, and inflamed mesenteries. E. Chicken and turkey ceca 10 days PI with *Histomonas meleagridis*. Note cecal cores (arrows). F. Discrete pathognomonic lesions with raised surface from a turkey infected with histomoniasis. G. Liver section showing histomonad PAS stain (arrows). H. Liver sections showing histomonads (arrow). H & E. ×1000. (Page)



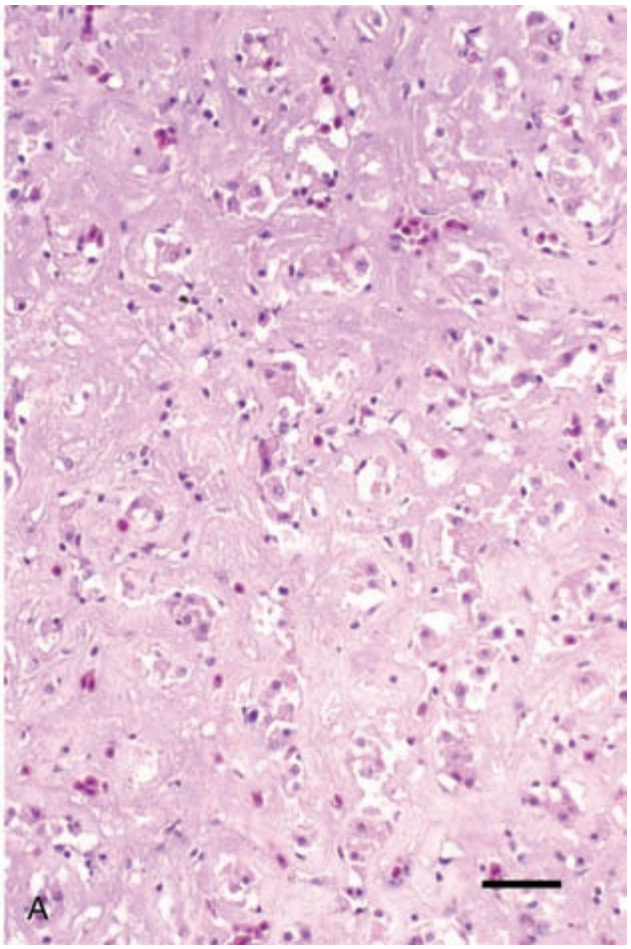
29.2. A–D. Vitamin A deficiency. A. Periorbital edema and lack of pigmentation. (Swayne) B. Squamous metaplasia of nasal mucosa. (Swayne, Barnes) C. Vitamin A deficiency. Distended, impacted mucosal glands resembling pustules in the esophagus. (Barnes) D. Squamous metaplasia has replaced all but a few focal areas of normal mucosa in the base of this esophageal gland. Distention has resulted from occlusion of opening and accumulation of keratin and cellular debris in the lumen. Inflammation resulting in formation of a pustule will occur if contents contact surrounding tissues. (Barnes) E–G. Rickets. E. Soft, thick ribs form a flattened thorax in this severely affected 8-day-old broiler chicken. Vertebrae are also short and thick. In less affected birds, enlargement at junctions of ribs with vertebrae and sternum, folding of sternal portions of caudal ribs resulting in a flat, broad thorax, and occasionally pathologic rib fractures may be seen. (Munger) F. Beak of affected chicken is soft and easily bent. (Swayne) G. Field or infectious rickets in turkeys occurs secondarily to intestinal disease. In this affected poult, there is excess, hypertrophic cartilage that is poorly vascularized because of a compression-induced fold fracture involving trabeculae at the physeal-metaphyseal junction. (Barnes) H. Osteopenia ("cage layer fatigue"). Pathologic fracture of rib with imperfect callous formation. There is minimal mineral being deposited at the fracture site. (Barnes)

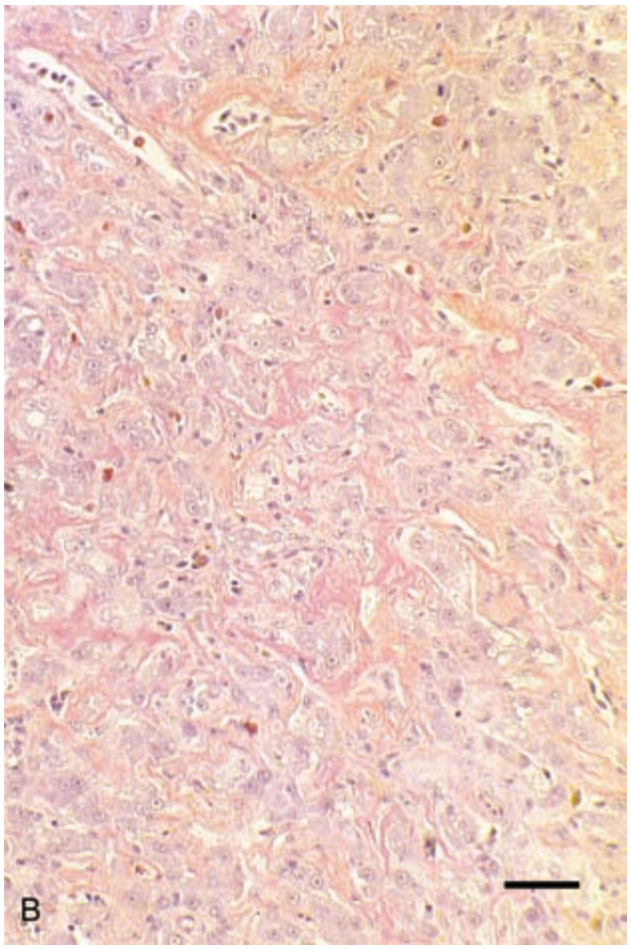


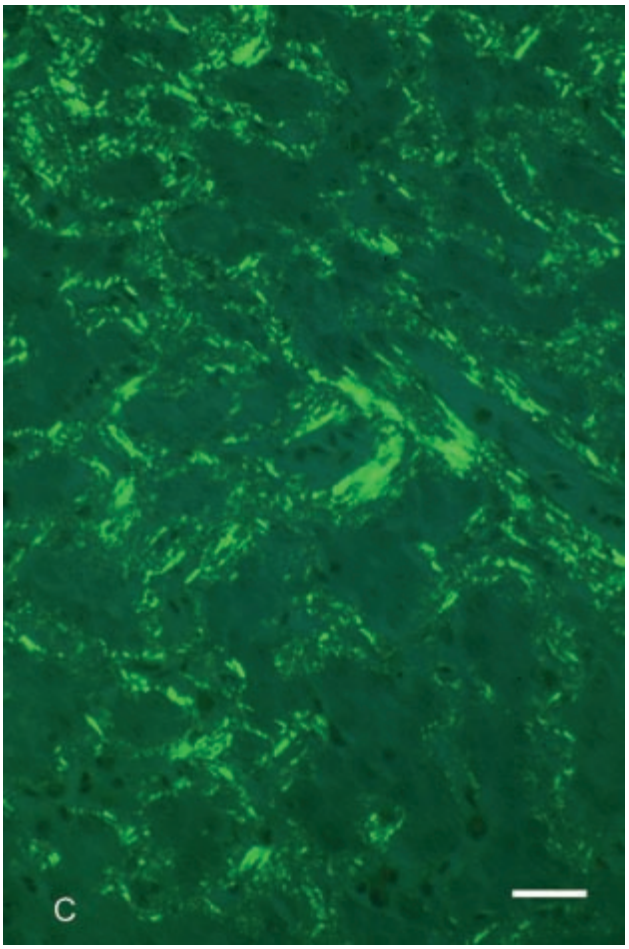
29.4. A–F. Nutritional encephalomalacia (vitamin E deficiency). A. Paresis in one poult and another with pronounced neurologic signs. While either clinical manifestation can be seen in turkeys, the latter is seen in chickens ("crazy chick disease"). (Barnes) B. Birds with neurologic signs have cerebellar swelling, edema, hemorrhage, and attenuation of folia. Coning of the swollen cerebellum into the foramen magnum is often seen. Lesions in the cerebrum also may occur but are not common. (Barnes) C. This bird with chronic nutritional encephalomalacia survived 3 days after onset of signs. Affected areas are now pale and shrunken. (Barnes) D. Severe malacia of cerebellum. Variable portions of affected outer folia are sharply separated from inner normal tissue. There is congestion and hemorrhage. At higher magnification characteristic fibrin thrombi in small vessels would be seen. Inflammatory cells are minimal to absent. (Barnes) E. Increased swollen astrocytes replace much of the normal cerebellar architecture in this bird with chronic encephalomalacia. Only isolated parts of the granular layer and individual Purkinje cells remain. (Barnes) F. Poults with paresis usually do not have brain lesions but have bilateral poliomyelomalacia as seen here. (Barnes) G. Nutritional myopathy. Degeneration of muscle fibers can result from inadequate vitamin E and/or selenium. These are seen as pale, often fusiform, linear streaks in skeletal muscle. Fibrosis, intramuscular fat deposition, and other myopathies can produce similar changes. (Barnes) H. Ventricular myopathy. Deficiency of vitamin E and/or selenium can produce myopathic changes in smooth muscle as well as cardiac and skeletal muscle. Lesions are seen as extensive, pale areas in ventriculus musculature. Turkeys are more commonly affected. (Munger)



30.5. Amyloid arthropathy in 32-wk-old brown chicken layers caused by *Enterococcus faecalis*. From left to right, normal control to most severely affected. There is accumulation of yellow-orange material (amyloid) in the lumen of the tibiotarsal joint. Note the articular cartilage is partially destroyed. (Barbara Daft)







30.6. Histologic section of liver with amyloidosis. A. Amyloid appears as homogenous eosinophilic material deposited extracellularly. Most hepatocytes have disappeared. H & E, bar = 65 µm. B. When amyloid is stained with Congo red it appears as brownish-orange under day light, Congo red, bar = 65 µm. C. Amyloid stained with Congo red emits apple green birefringence under polarized light, Congo red, bar = 30 µm.